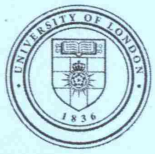

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Regulation of MDM2 Stability and Function by Signalling from mTOR/S6K Pathway

by

Mong-Lien Wang

A thesis submitted to the University College of London in fulfilment with the requirements for the degree of Doctor of Philosophy

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DECLARATION

I, Mong-Lien Wang, confirm that all the work presented in this thesis is the result of my own work and does not constitute part of any other thesis. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. The work herein described was carried out while I was a graduate student at University College London, Department of Biochemistry and Molecular Biology under the supervision of Prof. Ivan Gout.

Mong-Lien Wang

ABSTRACT

Ribosomal S6 kinase belongs to the AGC family of S/T kinases, which also include PKA, PKB/Akt, PKC, and SGK. The family of S6K consists of two isoforms, S6K1 and S6K2, which have cytoplasmic and nuclear splicing variants. Genetic and biochemical studies indicate that S6K is a key player in the regulation of cell growth, size, and the glucose metabolism. It has been also implicated in G1 to S transition of the cell cycle.

The MDM2 is another important regulator of controlling the cell cycle. It was initially identified as a negative regulator of p53, and several other targets of MDM2, such as $p21^{waf1/cip1}$, were consequently discovered. The MDM2 protein is generally believed to be involved in p53-dependent and –independent pathways, and the importance of its role on cell cycle regulation and tumor development have been highlighted. Recent studies indicated that PI3K/Akt signalling promotes the phosphorylation of MDM2 at Ser166 and Ser186, which triggers the downregulation of p53.

Here I present evidence that MDM2 is physically and functionally associated with S6K. Using mass spectrometry and phosphospecific antibody, I found that MDM2 is phosphorylated by S6K at Ser166. Phosphorylation of MDM2 at Ser166 closely correlates with the activation pattern of S6K when cells are treated with various mitogenic stimuli. Moreover, Ser166 is phosphorylated in cellular response to amino acid sufficiency, which is known to be mediated via the S6K, but not PKB/Akt pathway. Consistent with this data, Ser166 phosphorylation is highly sensitive to rapamycin, and in addition, mitogen-induced stability of MDM2 is inhibited in a dose-dependent manner by rapamycin. Interestingly, instead of regulating p53 transcriptional activity, I found that the S6K/MDM2 pathway signals to $p21^{waf1/cip1}$. Taken together, my study indicates that mTOR/S6K pathway signals to MDM2 and may thereby provide insight into how rapamycin potentially blocks the G1/S transition stage/phase of the cell cycle.

With Love to My Parents

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ABBREVIATIONS

AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Adenosine3`,5`-cyclic monophosphate
CBC	Cap-binding complex
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
cGMP	Guanosine3`,5`-cyclic monophosphate
DAG	sn-1,2-Diacylglycerol
DCS	Donor calf serum
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
dNTP	Deoxyribonucleoside triphosphate
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
DTT	Dithiotreitol
4E-BP1	Eukaryotic initiation factor-4E binding protein 1
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetra-acetic acid
eEF1A	Eukaryotic elongation factor 1A

eEF2k	Eukaryotic elongation factor 2 kinase
E2F	Early promoter 2 factor
EGF	Epidermal growth factor
EGTA	Ethyleneglycol-bis(β-aminoethyl)-N,N,N`,N`-tetraacetic acid
ERK	Extracellular signal-regulated kinase
EST	Expressed sequence tag
Fab	Antigen-binding fragment
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FKBP12	FK506-binding protein 12
FTI	Farnesyltransfarase inhibitor
GAP	GTPase-activating protein
GSK	Glycogen synthase kinase
HIF-1	Hypoxia-induced factor 1
HPLC	High pressure liquid chromatography
IEF	Isoelectric focusing
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor 1
IP_3	Inositol-1,4,5-triphosphate
IPG	Immobilized PH gradient
IRS-1	Insulin receptor substrate-1
LMB	Leptomycin B
MAPK	Mitogen-activated protein kinase
MDM2	Murine double minute 2
MEF	Mouse embryo fibroblast

MEK	MAPK kinase
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
mRNP	Messenger ribonucleic acid protein
mTOR/FRAP	Mammalian target of rapamycin/FK506-binding protein, rapamycin-associated protein
Ni-NTA	Nickel-nitrilotriacetic acid
NLS	Nuclear localisation signal
NES	Nuclear export signal
OD	Optical density
PAK	p21-activated kinase
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PDK1	3`-Phosphoinositide-dependent kinase-1
PDZ	Postsynaptic density-95, discs large, zona occludens-1
PEG	Polyethylene glycol
PH	Pleckstrin homology
PIF	PDK1-interacting fragment
PI3K	Phosphatidylinositol-3`-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLCγ	Phospholipase Cγ
PMA	Phorbol 12-myristate 13-acetate
PCR	Polymerase chain reaction

PP1, 4 and 6	Protein phosphatase1, 4 and 6
PP2A	Protein-serine/threonine phosphatase 2A
PRK2	PKC-related kinase 2
p90RSK	p90 Ribosomal S6 kinase
PtdIns(3)P	Phosphatidylinositol-3-phosphate
PtdIns(3,4)P_2	Phosphatidylinositol-3,4-bisphosphate
PtdIns(3,5)P_2	Phosphatidylinositol-3,5-bisphosphate
PtdIns(3,4,5)P_3	Phosphatidylinositol-3,4,5-trisphosphate
PTEN	Phosphatase and tensin homologue, deleted on chromosome 10
PVDF	Polyvinyldene difluoride
Rb	Retinoblastoma
RNA	Ribonucleic acid
RNAi	RNA inteferone
rpS6	Ribosomal protein S6
rRNA	Ribosomal RNA
S6K	Ribosomal protein S6 kinase
SDS	Sodium n-dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Ser	Serine
SGK	Serum- and glucocorticoid-induced protein kinase
SH3	Src homology 3
siRNA	Small interfering RNA
snRNA	Small nuclear RNA
SRE	Serum response element
TEMED	N,N,N`N`-Tetramethylethylenediamine

TetO	Tetracycline operator
TGFβ	Transforming growth factor β
Thr	Threonine
5`-TOP	5`-terminal oligopyrimidine tract
T-Rex	Tetracycline-regulated expression system
tRNA	Transfer RNA
TSC	Tuberous sclerosis complex
Tween 20	Polyoxyethylenesorbitan monolaurate

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CHAPTER ONE:

GENERAL INTRODUCTION

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1.1 Regulation of cell growth by signalling pathways

Growth, division, differentiation and death are the most fundamental aspects of cellular function. The magnitude of a multicellular organism depends mainly on the size and number of cells contained within it. Cell growth (increase in mass) and proliferation (increase in number) are tightly regulated to ensure that animals grow to a genetically defined dimension, and that their tissues are appropriately sized, precisely proportioned, and fully functional. The size of a cell in a human body can vary dramatically. For example, some neurons are nearly 1,000 times larger than epithelial or endothelial cells. Intrinsic and extrinsic factors are known to control the size of a cell. In many organisms, cell size is influenced by ploidy (the number of genome sets). In Drosophila, a polyploid salivary gland cell is up to 1000 times larger than a diploid cell. In addition to genetic controls, cell growth and proliferation are also influenced by external factors, such as nutrients and various stresses. Starvation not only extends doubling time in Drosophila cells, it also causes a reduction in final cell size. Obviously cells must grow before they proliferate, otherwise they would become increasingly smaller, a process that could not be sustained indefinitely.

The phrase 'cell growth' is used to describe an increase in cell volume, cell mass or biosynthetic rates. The regulation of cell growth is achieved by balancing anabolic and catabolic processes and by a number of signalling pathways that are regulated by growth factors, nutrients, and cellular energy status. Mammals are developmentally programmed to grow to a certain size as adults, and the physiological mechanisms which control cell, organ and organism size have been studied extensively in the last decade. These studies have led to the

identification of numerous signalling proteins and pathways with crucial positions in the regulation of growth. The importance of these pathways in cell biology is underscored by the fact that they either directly or indirectly influence cell survival and proliferation, growth and cell cycle progression, motility and differentiation.

Genetic studies in various multicellular organisms, mainly worms, flies and mice, have shown that Target of Rapamycin (TOR), phosphatidylinositol 3-kinase (PI3K), small GTPase Ras, and transcription factor Myc are key players in the regulation of cell growth (Coelho and Leevers, 2000;Harris and Lawrence, Jr., 2003;Bernard and Eilers, 2006;Ruvinsky and Meyuhas, 2006). It is now generally accepted that mTOR is a central controller of cell growth and that these functions are conserved from yeast to man (Schmelzle and Hall, 2000;De Virgilio and Loewith, 2006;Wullschleger *et al.*, 2006;Sandsmark *et al.*, 2007;Stipanuk, 2007). The molecular mechanism of signal transduction via mTOR involves sensing the level of nutrients and energy within a cell, and directing positive or negative regulation of anabolic pathways via the formation of signalling complexes and phosphorylation of downstream effectors, such as protein kinases Akt/PKB, ribosomal S6K, and translational inhibitor 4E-BP1. The most obvious example of an anabolic process regulated by mammalian TOR, is protein synthesis. Studies from other research laboratories indicate that mTOR positively controls protein synthesis at multiple levels. mTOR does this specifically by regulating ribosomal biogenesis, initiating translation, assembling the translation machinery, mRNA turnover and expressing and activating amino-acid and glucose transporters.

The second signal transduction pathway which coordinates biosynthetic pathways and cell growth involves PI3K signalling. Biochemical and genetic studies have provided evidence that PI3K pathway regulates cell growth in response to growth factors and hormones, and the activation of receptor tyrosine kinases. In this pathway, Class 1A PI3 kinases and tumor suppressor PTEN are the main coordinators of signalling events. Besides the control of cell growth, the PI3K

signalling cascade is known to regulate other cellular functions including cell survival, proliferation and migration in a context-dependent manner.

In addition to mTOR and PI3K signalling to growth, the MAP kinase module: Raf-MEK-ERK appears to additionally mediate the activation of biosynthetic processes, protein synthesis in particular. In this signalling cascade, extracellular signals activate via receptor tyrosine kinases, G-protein-coupled receptors or integrins such as small GTPase Ras, which induces phosphorylation and subsequent inactivation of tumor suppressor tuberous sclerosis complex 2 (TSC2) – a negative regulator of cell growth. Activated ERK1/2 and the MAPK-activated kinase RSK1 were shown to mediate inactivation of TSC2 by phosphorylating unique sites. Furthermore, loss-of-function mutations in the NF1-encoded tumor suppressor neurofibromin, a Ras-GAP, were shown to deregulate TSC2 and ultimately promote growth signalling (Johannessen *et al.*, 2005).

These signalling cascades control growth by synchronously regulating a large number of intracellular biological processes. The importance of interplay between mTOR, PI3K and MAPK pathways in controlling cell growth is underlined by numerous biochemical, mutational and genetic studies within the last decade. Cross-talk signalling between the PI3K, TOR and MAPK pathways, occurs through a number of positive and negative feedback loops. Collectively, normal cell growth is achieved in response to accurate and coordinated levels of signalling through these pathways.

Downregulation of the molecular mechanisms controlling cell growth results in cells of altered size and can also lead to developmental errors which may contribute to a variety of pathological conditions, including cancer and inflammatory or metabolic diseases. Therefore, key players in the cell growth regulation process are excellent targets for drug discovery and therapeutic interventions.

1.1.1 Signal transduction in growth control and the role of ribosomal S6 kinases

1.1.1.1 PI3K pathway

Signal transduction via PI3K was the first pathway to be implicated in the regulation of S6K activity. The link was initially established by studying molecular signalling via insulin and platelet-derived growth factor receptors (Chung *et al.*, 1994). Since this study was conducted, an overwhelming amount of biochemical, genetic and pharmacological data has been accumulated in support of PI3K as a crucial mediator of S6K activation in response to various mitogenic stimuli, including growth factors, hormones, cytokines and oncogene products. These studies led to the identification of secondary messengers which transduce the signal from activated PI3K to downstream effectors, including S6K (Engelman *et al.*, 2006).

Phosphatidylinositides-3-kinases (PI3Ks), constitute a lipid kinase family characterized by their ability to phosphorylate the inositol ring 3'-OH group in inositol phospholipids (Fruman *et al.*, 1998). The initial characterization of PI3K in its entirety, came through the purification and cDNA cloning of the enzyme from bovine brain tissue, and demonstrated that PI3K is made up of two subunits, p110 and p85 (Otsu *et al.*, 1991;Hiles *et al.*, 1992). Analysis of these proteins revealed that catalytic activity is a feature of the 110 kDa subunit, while the primary structure of the 85 kDa subunit suggested a regulatory role.

In the last decade, a wide range of proteins possessing PI3K activity have been identified. At present, mammalian PI3Ks can be divided into three distinct classes (class I, II and III), which differ in their subcellular and tissue distribution, substrate specificity and mechanisms of activation by extracellular agonists

(Wymann and Marone, 2005). The class I of PI3Ks is further divided into the subclass IA, which is activated by receptors with protein tyrosine kinase activity, and subclass IB, which is activated by receptors coupled with G proteins. Four isoforms of the p110 catalytic subunit (α, β, γ, and δ) and five regulatory subunits (p85α, p85β, p55γ, p101 and p84) have been described in the class I PI3K. The physiological substrate for receptor-coupled class I PI3Ks is phosphatidylinositol (4,5) biphosphate, and hence the primary product of their action is phosphatidylinositol (3,4,5) triphosphate (Hawkins *et al.*, 1992).

The subclass 1A and class III PI3Ks are most relevant to upstream regulation of S6K signalling. The first growth factor which was shown to activate PI3K was PDGF (Coughlin *et al.*, 1989;Kazlauskas and Cooper, 1990;Escobedo *et al.*, 1991). Since then, a large variety of membrane receptors have been shown to drive the activation of Class I PI3Ks either directly or via associated tyrosine kinases, G-protein βγ subunits or activation of Ras. In the case of PDGF receptor, ligand-induced receptor dimerization and autophosphorylation results in recruitment of PI3K activity by direct binding of the p85 SH2 domains to phosphorylated tyrosine in the YMXM motifs in the kinase insert region, situated in the intracellular portion of the receptor (Coughlin *et al.*, 1989;Escobedo *et al.*, 1991). The ability of p85 to bring the catalytic subunit to the activated tyrosine kinase receptors at the plasma membrane is a key event in class 1A PI3K signalling. Downregulation of this signalling event has a major impact on cellular functions. It has been shown that constitutive membrane targeting of p110 catalytic subunits of PI3K creates a constitutively active enzyme that generates PIP_3 independently of growth factor stimulation, leading to abnormal proliferation (Reif *et al.*, 1996). In addition to the shuttling function, the p85 regulatory subunit seems to have an inhibitory effect on p110 enzymatic activity. It has been proposed that mitogen-induced association of p85/p110 with ligand-activated receptors alleviates this inhibition (Kodaki *et al.*, 1994). In contrast to PDGF receptor, with which PI3K directly associates through its p85 regulatory subunit, the binding of insulin receptor and PI3K is mediated by IRS proteins, a family of adaptor proteins that are essential both for PI3K activation

and for mediating the effects of insulin (Araki *et al.*, 1994;Withers *et al.*, 1998;White, 2003).

Once the p110 catalytic subunit is targeted to the receptor activated complexes, it can phosphorylate its main substrate, phosphatidylinositide 4,5- biphosphate (PIP_2), and thereby generate phosphatidylinositide 3,4,5-triphosphoate (PIP_3), an important secondary messenger at the inner side of the plasma membrane (Hawkins *et al.*, 1992). PIP_3 mainly functions as a membrane anchor and allosteric regulator for key proteins in PI3K signal transduction, such as PKB/Akt and PDK1 (Figure 1.1). Both kinases associate with plasma membranes through Pleckstin homology (PH) domains, which specifically recognize and bind PIP_3 with high affinity. The translocation of PKB/Akt to the plasma membrane is crucial for its activation, as this brings the kinase into close contact with its activator, phosphoinositide-dependent protein kinase 1 (PDK1) (Stokoe *et al.*, 1997). Conformational changes also occur when PKB/Akt binds to PIP_3 at the plasma membrane, thus exposing the phosphorylation site and enabling PDK1 to phosphorylate and activate PKB/Akt (Alessi *et al.*, 1997b). Once activated, PKB/Akt mediates the activation and inhibition of several targets, resulting in cellular survival, growth, and proliferation through various mechanisms (Coelho and Leevers, 2000).

Signalling via the PI3K pathway has long been implicated in the transduction of mitogenic information to the S6K cascade. The first clue to this link originated from analysis of PDGF-R mutants, defective in PI3K signalling (Chung *et al.*, 1994). The activation of S6K1 (known at that time as p70 S6K) was found to be abrogated in downstream signalling from kinase-dead (K635R) and PI3K-defective (Y740F/Y751F) PDGF-R mutants. Analysis of S6K signalling in cells overexpressing constitutively active p110 or dominant negative forms of p85 and lacking the p110 binding site, further strengthened the critical role of PI3K signalling in mitogen-induced S6K activation (Burgering and Coffer, 1995). Further links between PI3K and S6K signalling were found from studies involving PI3K specific inhibitors, wortmannin and LY294002

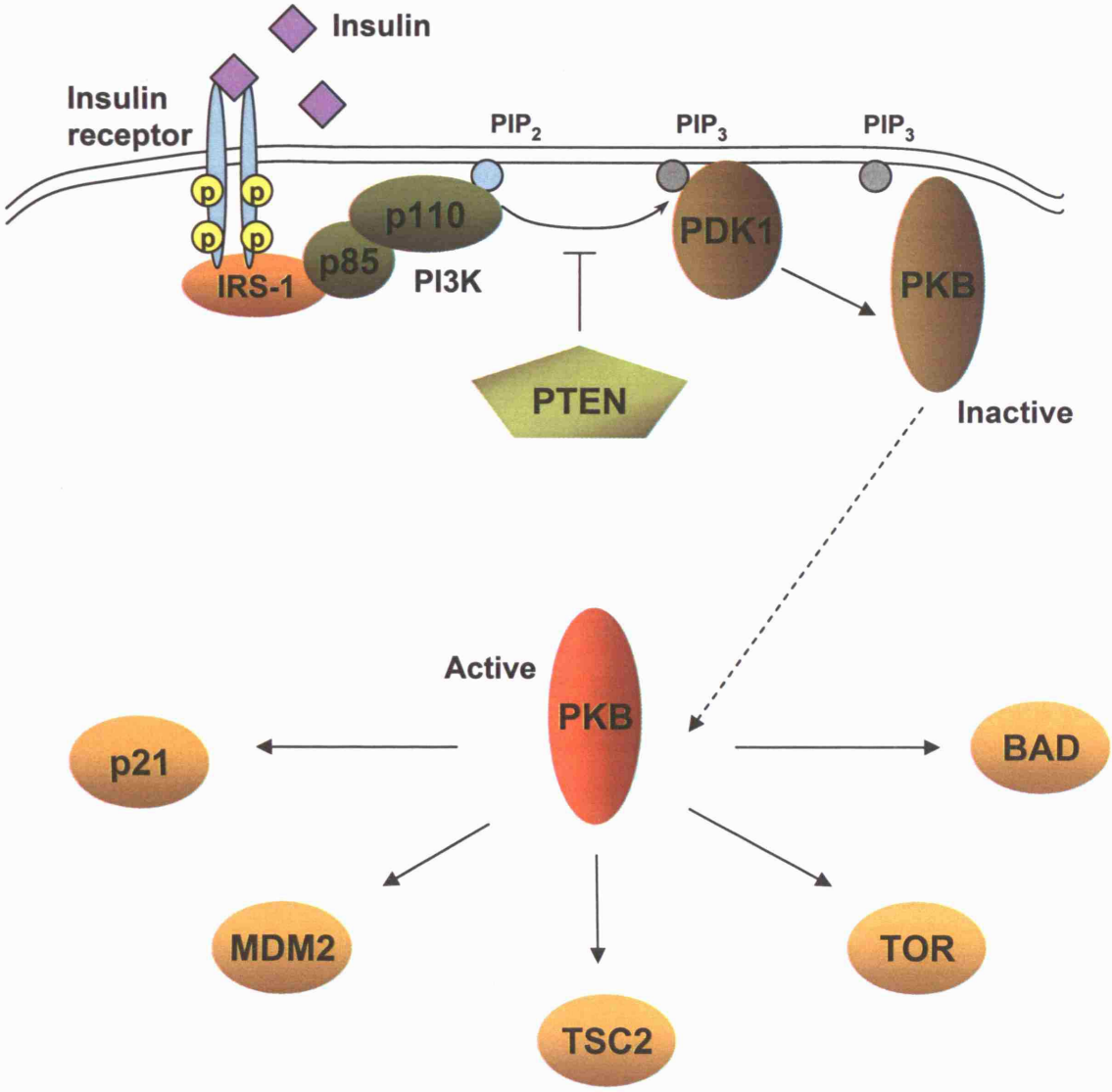


Fig. 1.1 Schematic representation of the PI3K/PKB signalling pathway.

The type IA PI3K signalling pathway is initiated by insulin or growth factors stimulation, which induce activation of receptor tyrosine kinases and the formation of multi enzyme complexes, involving IRS-1 and the PI3Ks. After binding to IRS-1, PI3Ks phosphorylate the 3'OH group on the inositol ring of PIP_2, converting it into PIP_3. PIP_3 consequently activates PDK1 and PKB. Activated PKB has diverse cellular functions through interaction and phosphorylation of different downstream proteins, such as p21, MDM2, TSC2, TOR, and BAD. Tumor suppressor PTEN inhibits PI3K signalling by dephosphorylating PIP_3. RTK, receptor tyrosine kinase; PIP_2, phosphotidylinositol diphosphate; PIP_3, phosphotidylinositol triphosphate; P, phosphate.

(Cheatham *et al.*, 1994;Myers, Jr. *et al.*, 1994;Monfar *et al.*, 1995). Both inhibitors were found to strongly inhibit S6K1 activity in cellular response to various mitogenic stimuli. *In vitro* studies indicated that wortmannin and LY294002 did not directly inhibit S6K activity. A number of wormannin/LY294002 sensitive sites have been identified in S6K1, including Thr229, Thr389, Ser404 and Ser411 (Weng *et al.*, 1998). Mutational analysis of these sites indicated that phosphorylation of Thr229 and Thr389 is necessary for full activation of S6K1. Phosphorylation of S6K1 at Thr229 was later found to be mediated by PDK1 which culminates multistage phosphorylation events, resulting in full kinase activation.

Recent studies have also implicated class III PI3K in regulatory signalling to S6Ks (Byfield *et al.*, 2005;Nobukuni *et al.*, 2005). Both laboratories presented evidence that class III PI3K hVPS34 mediates signal transduction to mTOR in response to amino acid stimulation. The loss-of-function RNAi experiments, overexpression of wild type and dominant negative mutants of hVPS34, convincingly demonstrated positive regulation of S6K1 activity by class III PI3K in response to amino acid availability. The established link will be further discussed in the section on the mTOR pathway (section 1.1.1.2).

S6K is generally accepted as being responsive to stimulation from the PI3K pathway, however recent studies have established S6K as a negative regulator of this pathway. These studies uncovered a feed-back regulatory loop, involving S6K and IRS1 (Insulin Receptor Substrate 1), a key player in insulin and IGF1 signalling. In a pioneering study, Haruta and colleagues showed that a PI3K- and rapamycin-sensitive pathway is involved in an inhibitory mechanism that impinges on the insulin-PI3K signalling pathway (Haruta *et al.*, 2000). They found that prolonged exposure to insulin leads to a decrease in both electrophoretic mobility and the level of IRS-1 protein, as well as PKB/Akt activity, while wortmannin (a PI3K inhibitor) and rapamycin (an mTOR inhibitor) can prevent the prolonged effect of insulin. Further studies confirmed the original results of this study and reported a decrease in IRS-1 protein in response

to prolonged insulin treatment (Tremblay and Marette, 2001;Zhang *et al.*, 2002;Berg *et al.*, 2002;Pirola *et al.*, 2003). Notably, the presence of amino acids was previously shown to decrease the activity of PI3K after insulin treatment, an effect that is associated with both an increase in IRS-1 phosphorylation and a reduction in IRS-1 stability (Patti *et al.*, 1998;Tremblay and Marette, 2001;Zhang *et al.*, 2002).

Specific Ser/Thr phosphorylation of IRS-1 can impair insulin-induced tyrosine phosphorylation of IRS-1, negatively affecting function in at least two ways: a) it can disrupt interaction with the insulin receptor and b) it can destabilize IRS-1 (Paz *et al.*, 1997;Greene *et al.*, 2003). A role of mTOR signalling in the phosphorylation of Ser307 at IRS-1 has been designated by the demonstration that treatment with rapamycin abrogates the phosphorylation of Ser307 induced by insulin (Gual *et al.*, 2003a;Gual *et al.*, 2003b;Carlson *et al.*, 2004). Furthermore, S6K has also been found to phosphorylate IRS-1 at Ser302. The phosphorylation at Ser302 was increased in the absence of TSC1-TSC2 function and is inhibited by the RNAi of S6K1 (Harrington *et al.*, 2004). These findings strongly indicate that a major form of negative feedback inhibition of PI3K results from activated growth signalling involving both the mTOR and S6K pathways.

1.1.1.2 mTOR signalling to S6K

The connection between mTOR and S6K signalling was initially established in studies employing the mTOR specific inhibitor rapamycin. These studies led to the identification of several rapamycin-sensitive phosphorylation sites in S6Ks, which contribute to the activation process in response to various extracellular stimuli. The mTOR kinase was shown to phosphorylate Thr389 directly, a key phosphorylation event in S6K1 activation. Molecular and genetic approaches provided further evidence, and uncovered a whole range of secondary messengers which coordinate signal transduction in the mTOR/S6K pathway (Figure 1.2).

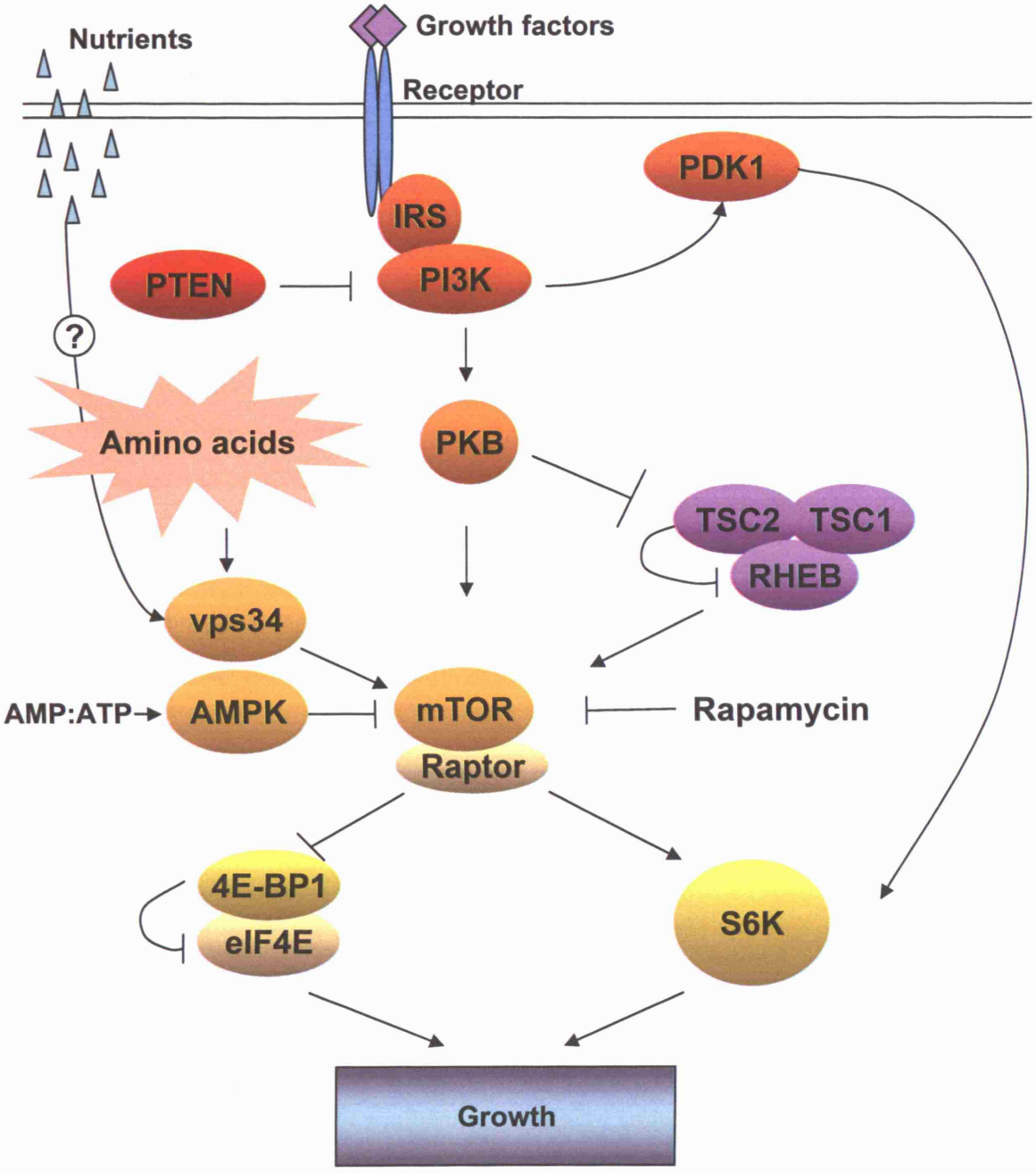


Fig. 1.2 Schematic representation of the mTOR/S6K signalling pathway.

Numerous extracellular and intracellular signals were shown to control the mTOR/S6K pathway. A whole range of mitogens, including growth factors, hormones, and cytokines trigger the activation of mTOR. Nutrients activate mTOR via class III PI3K (vps34), while class IA PI3K transduces the activation signal to mTOR via PKB. AMPK senses the level of ATP in the cell and exhibit negative regulation on mTOR by activating tumor suppressor TSC1/2. TSC2 inhibits mTOR functions through binding to and repressing Rheb. Rapamycin is a specific inhibitor for mTOR kinase activity. Activated mTOR phosphorylates and regulates the function of its two main downstream effectors, S6K and 4E-BP1, which mediate the regulation of translation and cell growth.

The Tor kinase was originally discovered as a specific inhibitory target of the anti-fungal, bacterial microlide, rapamycin which exhibits a strong immuno-suppressive activity (Calne *et al.*, 1989;Pohanka, 2001;Saunders *et al.*, 2001). Later investigations revealed that mTOR is a serine/threonine kinase, belonging to a large family of PI3K-related kinases, which also include DNA-dependent protein kinase, ATM and ATR (Burnett *et al.*, 1998a). Rapamycin is not the direct inhibitor of Tor kinase activity. The inhibitory effect of rapamycin is achieved indirectly by forming a gain-of-function complex with the immunophilin FKBP12, which binds to and attenuates mTOR activity. The regulation of mTOR kinase activity has been studied extensively in the last decade. These studies led to the identification of S6K and 4E-BP1 (eIF4E binding protein 1) as major downstream targets of mTOR kinase activity (Sandsmark *et al.*, 2007). The activation of S6K and phosphorylation of 4E-BP1 at specific sites have been used as the readouts of mTOR signalling. In addition, a wide range of cellular proteins with various enzymatic activities, scaffold and adaptor functions, have been implicated in signalling to - and regulating the activity of - mTOR. These include protein kinases PKB/Akt and AMPK, tumor suppressors PTEN and tuberous sclerosis complex TSC1/2, small GTP-binding protein Rheb and scaffolding proteins Raptor and Rictor. Numerous extracellular and intracellular signals have been shown to control mTOR activity. A variety of mitogens, including growth factors, hormones and cytokines, trigger activation of the mTOR pathway (Jacinto and Hall, 2003;Kim and Novak, 2007). In contrast to other major signalling cascades, the mTOR pathway has been recognized as a critical checkpoint in nutrient availability, especially amino acids. Moreover, the level of cellular energy (AMP:ATP ratio) is sensed by the mTOR pathway. By deciphering signalling information from mitogens, and detecting nutrient and energy availability within the cell, mTOR coordinates the induction of biosynthetic processes, such as protein synthesis, through the phosphorylation of key downsteam messengers.

Although the TOR pathway is regulated by mitogens, unlike the Ras and PI3K pathways, it contains no domains which enable protein targeting to the

cytoplasmic membrane, where the other signalling pathways obtain growth factor-mediated information and transduce it to downstream messengers. Therefore, the activation cascade for TOR signalling requires upstream regulators in response to growth factor stimulation. Pharmacological and genetic studies have placed the TOR pathway downstream of PI3K signalling and/or in a parallel pathway. In mammalian cells, ligand binding to various growth factor receptors results in PI3K activation, as described above. This, in turn, triggers a number of effectors which transduce signalling information to the TOR pathway. Although the mechanism by which TOR is regulated by PI3K or its downstream effectors is not fully understood, the key players within this signalling crosstalk have been identified. For instance, PKB/Akt has been shown to activate the TOR cascade by two different means, involving direct and indirect regulation. Studies from Shepherd's laboratory on the signalling of PI3K to mTOR, led to the identification of two PKB/Akt phosphorylation sites on mTOR, Ser2448 and Ser2446 (Nave *et al.*, 1999;Cheng *et al.*, 2004b). It was shown that insulin stimulates Ser2448 phosphorylation via PKB/Akt, which is attenuated by amino acid starvation (Nave *et al.*, 1999). Recently, the phosphorylation of mTOR at Ser2448 was shown to also be mediated by S6Ks via a feed-back regulation (Holz and Blenis, 2005;Chiang and Abraham, 2005). It has been proposed that mTOR phosphorylation at this site allows the activation of its kinase activity. The phosphorylation of Ser2246 by PKB/Akt is mediated by nutrient availability, but insulin signalling inhibits phosphorylation of this site (Cheng *et al.*, 2004b). The indirect effect of PKB/Akt on mTOR involves phosphorylation of the tuberous sclerosis complex, TSC1-TSC2, shown to have an inhibitory effect on TOR signalling (Potter *et al.*, 2001;Gao and Pan, 2001;Gao *et al.*, 2002).

Numerous studies have indicated that both the metabolic state of the cell, and the availability of nutrients regulate S6K1 activity through the mTOR signalling pathway. A pathway which allows amino acids to regulate the kinase activity of mTOR, has only recently been identified. It was found that amino acids play an essential role in maintaining a basal level of mTOR signalling and its ability to respond to mitogenic stimuli. Many types of mammalian cells undergo rapid

dephosphorylation of S6K and 4E-BP1 when deprived of amino acids, and these cannot be fully rephosphorylated following the addition of insulin, which normally causes phosphorylation of both proteins. Readdition of amino acids restores the mTOR signalling to S6K and 4E-BP1 and its ability to respond to insulin (Hara *et al.*, 1998;Proud, 2002). A major breakthrough came from the identification of class III PI3K hVPS34 as a key upstream regulator of mTOR signalling (Byfield *et al.*, 2005;Nobukuni *et al.*, 2005;Nobukuni *et al.*, 2007). It is well known that hVPS34 uses phosphatidylinositol to produce the monophosphate signalling lipid PI(3)P. An increase in PI(3)P production in cells stimulated with amino acids, but not with growth factors or mitogens, was observed with the use of confocal microscopy and monoclonal antibody specific for PI(3)P. In addition, an *in vitro* PI3K assay confirmed the induction of hVPS34 activity in cellular response to amino acid availability. The activity of S6K1 was shown to be positively regulated by dose-dependent overexpression of hVPS34 in cells stimulated with amino acids. The loss-of-function RNAi experiments added more weight to the signalling link, which had already been established. The RNAi of hVPS34 were shown to completely block both the production of PI(3)P and the activation of S6K1 in response to amino acids.

The upstream regulation of hVPS34 in response to amino acid availability is not yet fully understood. Evidence exists for both intracellular and/or membrane bound sensors for amino acids as mediators of mTOR activation.

Recent studies have indicated that mTOR also functions as a checkpoint by which cells can sense and decode changes in energy status, which in turn determine the rate of cell growth and proliferation (Jacinto and Hall, 2003). The ability of insulin to activate mTOR becomes impaired upon a reduction in cellular ATP levels by a reduction in glucose availability or inhibition of mitochondrial respiration, suggesting that cellular energy has a direct impact on mTOR activity (Dennis *et al.*, 2001). A novel pathway which senses cellular ATP levels and transduces the information to mTOR has been discovered recently. AMP kinase (AMPK) was placed in the pathway as a key sensor of the AMP:ATP ratio within

the cell (Kimball, 2006;Inoki *et al.*, 2006). An increase in the level of AMP causes AMPK activation, thus targeting downstream regulators of biosynthetic pathways. AMPK-mediated phosphorylation of tumor suppressor TSC2 activates its GAP activity towards a small GTPase Rheb which is a positive regulator of mTOR. Inactivation of Rheb downregulates mTOR activity and downstream signalling, including phosphorylation of S6K and 4E-BP1 (Garami *et al.*, 2003;Inoki *et al.*, 2003a).

In summary, mTOR signalling gives rise to a second branch of regulatory events which control the activity of S6Ks, mainly via nutrient and energy sufficiency. It is important to note that full activation of S6K requires the contribution of both PI3K and mTOR pathways, which deliver signalling information from growth factors/mitogens and nutrients/energy sufficiency, respectively.

1.1.1.3 MAP/Erk Kinase pathway

The mammalian mitogen-activated protein kinase (MAPK) pathway, consisting of Ras, Raf, Mek, MAP/Erk kinase, is an information highway for the transmission of extracellular signals from the cell surface to the nucleus (Figure 1.3). It begins from the activation of a variety of growth factor receptors specific for the platelet-derived growth factor (PDGF), the epidermal growth factor (EGF), the vascular endothelial growth factor (VEGF), and the fibroblast growth factor (FGF) etc (Rossomando *et al.*, 1989). Following ligand binding, growth-factor receptor tyrosine kinases become activated, leading to the association of adaptor proteins and consequently, activation of Ras. Ras is a small membrane-bound guanine nucleotide-binding protein that acts as a molecular switch linking receptor tyrosine kinase activation to downstream signalling events. Ras cycles intracellularly between an active GTP-bound form and an inactive GDP-bound form. The activity of Ras is regulated by the opposing activities of guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). In its GTP-bound form, Ras activates the kinase activity of Raf, a serine/threonine

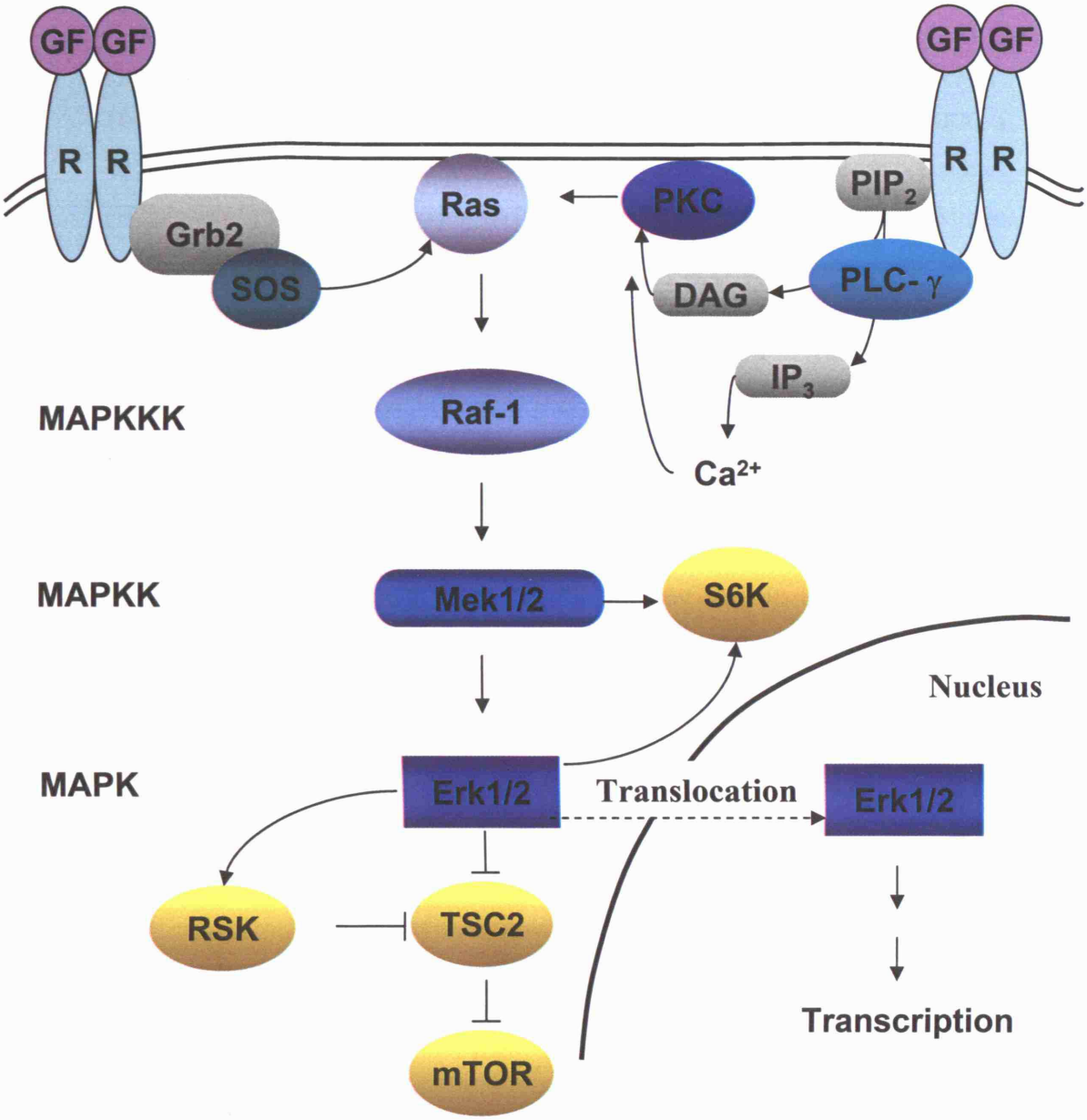


Fig. 1.3 Regulation of S6K signalling via MAP signalling pathway.

The mammalian mitogen-activated protein kinase (MAPK) pathway is information highway for the transmission of extracellular signals from the cell surface to the cell nucleus. It begins from the activation of growth factor receptors, which leads to the association of adaptor proteins and activation of small GTPase Ras. Ras activates Raf, a serine/threonine kinase, and its downstream signalling cascade. Activated Raf forms a complex with Mek and phosphorylates it, which consequently activates Erk. Erk dimmerizes and translocates to the nucleus, where it involves in transcription regulation. Both MEK and Erk1/2 were found to phosphorylate and activate S6K1/2. In addition, MAPK-activated kinase RSK inhibits the function of TSC1/2 complex by phosphorylation of TSC2. GR, growth factor; R, growth factor receptor; PLC-γ, phospholipase C-γ; PIP_2, phosphotidylinositol-4,5-diphosphate; DAG, diacylglycerol; IP_3, inositol-1,4,5-triphosphate; PKC, protein kinase C.

kinase, and its downstream signalling cascade (Howe *et al.*, 1992;Dickson *et al.*, 1992;Avruch *et al.*, 1994). Activated Raf forms a stable complex with MEK, a 45 kDa dual-specificity kinase, and phosphorylates MEK on serine residues (Huang *et al.*, 1993). Following Raf phosphorylation, the dual-specificity kinase, MEK, consequently activates MAP kinase, also known as extracellular signal-regulated kinase (Erk), by phosphorylation of both tyrosine and threonine residues (Crews and Erikson, 1992;Crews *et al.*, 1992). An activated Erk dimerizes and translocates to the nucleus where it phosphorylates a variety of transcription factors responsible for the regulation of gene expression and the promotion of cell proliferation (Lin *et al.*, 2001;Cheng *et al.*, 2004a).

The regulation of cell growth via the MAPK pathway is mediated in several ways. A signalling connection between the MAPK and mTOR/S6K pathways has been demonstrated in a number of studies. For example, the activation of a major downstream target of mTOR, S6K, is dependent on the MAPK pathway. Both isoforms of S6K:- S6K1 and 2, possess, at the C-terminus, conserved proline-directed sites, whose phosphorylation is mediated through the MAPK pathway. Cross-talk between ERK and S6K signalling was found in different experimental settings, including primary adult cardiomyocytes and hypertrophic cardiac growth, small cell lung cancer cells and NIH 3T3 adipocytes (Lenormand *et al.*, 1996;Herbert *et al.*, 2000;Wang *et al.*, 2001b). In these studies, *in vivo* activation of either c-Raf or MEK alone appeared to be sufficient for S6K activation.

Recently, extracellular signal-regulated kinase (Erk) has been found to play an important role in the posttranslational inactivation of TSC2. Studies from the Pandolfi laboratory demonstrated that Erk-dependent phosphorylation of TSC2 at Ser 664 disrupted the TSC1-TSC2 complex, markedly impairing its stability and ability to inhibit mTOR signalling, cell proliferation, and oncogenic transformation (Ma *et al*, 2005). These findings positioned the Ras/MAPK pathway upstream of the TSC1/2 tumor suppressor complex and suggested that Erk may modulate mTOR signalling and contribute to disease progression through

phosphorylation and inactivation of TSC2.

Furthermore, the MAPK-activated kinase RSK1, has been also implicated in the regulation of mTOR pathway via specific interaction and inactivation of the phosphorylation of TSC2 (Roux *et al.*, 2004). In this study, RSK1 was shown to phosphorylate TSC2 Ser-1798, a regulatory site located at the highly conserved C terminus. In a phosphorylated state, the tumor suppressor functions of the TSC1/2 complex are impaired, resulting in increased mTOR signalling to S6K1.

An additional regulatory link between the mTOR growth controlling pathway and Ras signalling involves tumor suppressor NF1. Johannessen and colleagues demonstrated that mTOR is constitutively activated in cells deficient for tumor suppressor NF1, which functions as a Ras-GTPase activating protein (Johannessen *et al.*, 2005). Loss-of-function mutations in the *NF1* gene result in the deregulation of Ras signalling. It was also shown that cells derived from NF1 patient tumors exhibit a constitutive phosphorylation of TSC2, directed by PKB/Akt. Importantly, *NF1*-deficient malignant human tumor cell lines are exquisitely sensitive to rapamycin. Furthermore, suppression of Ras activity significantly attenuates mTOR activation in response to lysophosphatidic acid (LPA) and insulin but has little or no effect on mTOR activation in response to platelet-derived growth factor (PDGF). These findings clearly demonstrate that Ras plays a critical role in the activation of mTOR in both normal and tumorigenic settings and suggest that rapamycin, or its derivatives, may represent a viable therapy for NF1.

The functional significance of the MAP kinase pathway is highlighted by the fact that several components of the pathway are oncogenic in their constitutively active forms. Given the high frequency of Ras oncogene activation in several common human cancers, its signal pathway is an important target for drug discovery. In actual fact, Ras and Raf were originally identified as viral oncogenes before their cellular counterparts were discovered (Chang *et al.*, 1982;Bonner *et al.*, 1985). Recent studies have additionally illustrated that

expression of activated Ras in normal murine bone-marrow cells was sufficient to induce cancerous transformation (Guo *et al.*, 2005). MEK mutants with constitutive activity have also been found to induce neuronal differentiation of PC12 cells and oncogenic transformation of fibroblast cell lines (Cowley *et al.*, 1994). These findings suggest the importance of the maintenance of correct function of MAPK signalling in normal cells, and the potential for targeting deregulated players of this signal transduction pathway for therapeutic use in the treatment of some forms of cancer.

1.2 The family of ribosomal S6 kinases

1.2.1 S6K – a member of AGC family of S/T kinases

The ribosomal S6 protein kinases (S6K) belong to the AGC superfamily of serine/threonine protein kinases (so named because it includes protein kinase A, G, and C), which include the PKB/Akt, PDK1, SGKs, and p90 RSK (Peterson and Schreiber, 1999;Miranda-Saavedra and Barton, 2007). The AGC kinases are well known as key components of the signal transduction machinery and members of this family mediate cellular responses to growth factors, hormones, nutrient sufficiency and various stresses. As well as a high degree of sequence similarity within the catalytic domains, AGC kinases also share a number of features concerning the way in which they are regulated. Each family member has a highly conserved phosphorylation site within the consensus sequence T(F/L)CGT, and full kinase activity depends upon phosphorylation of its catalytic domain activation loop. Several family members also possess a second conserved motif outside the catalytic domain that is approximately 160 amino acids in length, from the carboxyl-terminal to the activation loop phosphorylation site. In S6K, PKB/Akt, and PKC, this carboxyl-terminal site contains the consensus sequence FXXF(S/T)(Y/F), where X indicates a non-conserved residue, and phosphorylation at this site is required for protein stability and/or kinase activity. With few exceptions, phosphorylation of both the activation loop site and the carboxyl-terminal site are critical for full activation of the AGC kinases. In the case of S6K1, the activation loop site and the carboxyl-terminal site have been mapped to Thr229 and Thr389 respectively (Pearson *et al.*, 1995;Dennis *et al.*, 1998).

1.2.2 S6K isoforms and their subcellular localization

There are two isoforms of S6K in mammals, the S6K1 and the later identified S6K2, which are the products of distinct genes (Gout *et al.*, 1998;Saitoh *et al.*, 1998;Lee-Fruman *et al.*, 1999). Two alternatively spliced isoforms have been identified for both, S6K1 and S6K2 (Figure 1.4). The difference between two splicing forms of S6K1, the 502-amino acid isoform (known as p70S6K1 or S6K1 II) and the 525-amino acid isoform (p85S6K or S6K1 I), resides at the amino terminus, where S6K1 I contains a 23 amino acid extension that carries a polybasic nuclear localization motif consisting of six consecutive arginine residues immediately following the initiator methionine residue (Coffer and Woodgett, 1994). Similarly, the two alternative start codons in the S6K2 are responsible for the expression of two splice variants of the kinase (Gout *et al.*, 1998). The S6K2 I and S6K2 II isoforms consist of 495 and 482 amino acids respectively, and the primary sequence of the two isoforms are almost identical, the only difference being the first 13 amino acids at the N-terminus of S6K2 I.

S6K1 II is the most thoroughly studied S6 kinase, and it is known to localize predominantly to the cytoplasm. However, it appears that this kinase shuttles between nucleus and cytoplasm because leptomycin B (LMB, a nuclear export inhibitor) treatment of cells causes accumulation of S6K1 II in the nucleus (Coffer and Woodgett, 1994;Reinhard *et al.*, 1994;Kim and Chen, 2000). Unlike S6K1 II, the S6K1 I isoform localizes exclusively in the nucleus due to the nuclear localization sequence located within its amino-terminal extension (Reinhard *et al.*, 1994). Similarly to S6K1 I, the 13 amino acid extension at the N-terminus of S6K2 I, contains a functional nuclear localization signal (NLS) which determines its constitutive nuclear localization. In contrast to S6K1 II, the S6K2 II isoform localizes predominately to the nucleus due to presence of NLS at the carboxyl-terminus (Lee-Fruman *et al.*, 1999;Koh *et al.*, 1999). Both S6K1 isoforms and S6K2 II were shown to be ubiquitously expressed with similar patterns of expression in various cell lines and tissues (Gout *et al.*, 1998;Saitoh *et al.*, 1998). However, S6K2 I exhibits differential and limited expression and is

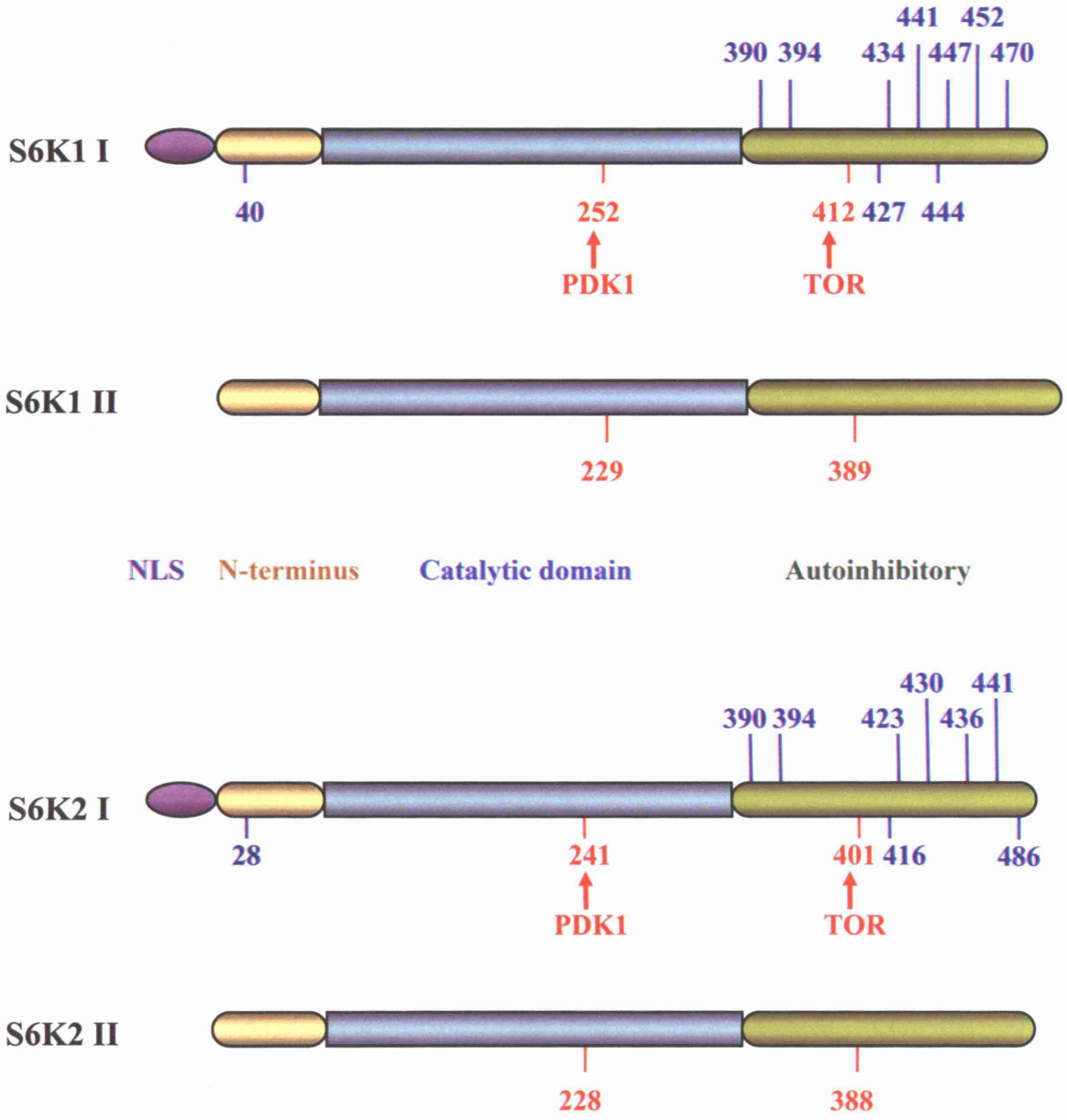


Fig. 1.4 Domain organization and the phosphorylation sites in S6K1/2.

Two classes of S6K encoded by different genes, S6K1 and S6K2, are shown in the figure. Both classes of S6K can be further divided into two isoforms, the nuclear form (I) and the cytoplasm form (II), due to different mRNA splicing. S6K1/2 possess 3 functional domains: the regulatory N-terminal domain, the catalytic domain, and the autoinhibitory domain. The nuclear forms of S6Ks have additional nuclear localization sequences (NLS) at their N-terminal. S6K1 and S6K2 have a very high level of overall sequence similarity with the greatest homology in the catalytic domain. Phosphorylation sites, as indicated in the figure, are mainly located in the C-terminal autoinhibitory domain. Both Ser252 and 412 in S6K1 I and the Ser241 and 401 in S6K2 I are critical for S6K activation.

not found in many tissues normally expressing other S6Ks (Minami *et al.*, 2001). This may suggest that S6K2 I regulates cellular processes distinct from other isoforms of S6K, whereas S6K1 I, S6K1 II, and S6K2 II share some common functions.

1.2.3 Domain organization of S6K

Excluding the 23 amino acid extension, both S6K1 I and S6K1 II can be divided into four functional domains: i) the amino-terminal segment of 65 amino acid residues; ii) the protein kinase catalytic domain; iii) the kinase extension or linker domain and iv) the carboxyl-terminal regulatory segment of 104 residues (Figure 1.4).

The amino-terminal noncatalytic domain is characterized by the presence of a highly acidic sequence, containing mainly aspartic or glutamic amino acids, while basic residues are absent. This motif is thought to interact with a basic carboxyl-terminal segment, locking the kinase in an inactive state (Banerjee *et al.*, 1990). The catalytic and kinase-extension domains are the main features that classify S6K as a member of the AGC kinase family and determine similar mode of activation. One unique attribute of the carboxyl-terminal segment of S6K1 is the presence of a putative autoinhibitory pseudosubstrate region. This region constitutes a moderately basic serine/threonine rich sequence whose composition is similar to the carboxyl-terminal region of S6 protein itself, which is the main physiological substrate for S6K and contains six phosphorylation sites at the C-terminus (Chan and Wool, 1988;Krieg *et al.*, 1988). Mitogen-induced phosphorylation of the autoinhibitory pseudosubstrate region is the initial regulatory event in the activation process of S6K mediated by multiple phosphorylation reactions. It causes the dissociation between the N- and C-terminal regulatory regions, exposing the kinase and kinase extension domains for further phosphorylations and allowing access to the substrate(s).

The overall sequence of S6K2 is very close to that of S6K1 with 70% identity and 85% similarity at the protein level, sharing the same domain organization as described above (Gout *et al.*, 1998). The catalytic domains of S6K1 and S6K2 share 83% identity at the amino acid level (Gout *et al.*, 1998). The kinase extension domain and the autoinhibitory pseudosubstrate domain are also very similar, with 80% and 73% identical, respectively. The major differences lie in the extreme N- and C-terminal regions. In contrast to other protein kinases, S6K1 and S6K2 lack canonical protein-protein interaction modules, which can drive them to the multienzyme complexes formed around activated receptors (via SH2, SH3, PTB, WW domains), or enable specific interaction with cytoplasmic membranes (via PH domain). However, the regulatory regions of both kinases contain recognition motifs which have been also implicated in protein-protein interactions. These include a PDZ motif and a proline-rich sequence at the C-termini of S6K1 and S6K2 respectively. These motifs may direct the kinases to distinct compartments or to different molecular complexes through interactions with proteins possessing PDZ and SH3 domains (Gout *et al.*, 1998). For example, the C-terminal PDZ binding motif recruits S6K1 to the actin cytoskeleton via binding to neurabin PDZ domain (Burnett *et al.*, 1998b). Furthermore, specific interactions between S6K2 and SH3 domains from Frg and Src tyrosine kinases has been observed in GST pull-down assay (T. Valovka, unpublished observation). These interactions are possibly mediated via C-terminal proline-rich sequences, known to form specific binding motifs for SH3 domains.

1.2.4 Signalling pathways leading to S6K activation

Activation of S6K is a complex process requiring multiple signalling inputs and it is likely that these inputs will be shared within isoforms. Recent research in this area has indicated that PI3K and mTOR pathways lie in parallel and converge on S6K, with amino acid and energy sufficiency mediated primarily by mTOR and growth factor/mitogen signalling being mediated mainly via PI3K.

For example, the activity of S6K1 is increased by expression of constitutively active class 1a PI3K (Weng *et al.*, 1995;Klippel *et al.*, 1996), while activation of S6K is blocked by dominant negative forms of the PI3K adapter/regulatory subunit, and by wortmannin and LY 294002 (Weng *et al.*, 1995;Moule *et al.*, 1995), inhibitors for PI3K activity. PKB/Akt, the downstream effecter of PI3K, is also proven to play a role in the activation process as the expression of constitutively active PKB/Akt activates S6Ks (Burgering and Coffer, 1995;Welch *et al.*, 1998).

S6K is indisputably a key element of the TOR signalling process, as rapamycin inhibits activation of S6Ks in response to all known stimuli, and the drug reduces ribosomal S6 protein phosphorylation in many cell types (Chung *et al.*, 1992). Overexpression of the rapamycin-resistant form of mTOR facilitates activation of S6K1 in cells incubated with rapamycin (Brown *et al.*, 1995). Furthermore, depletion of mTOR with short-interfering RNA (siRNA) inhibits activation of S6K1 (Kim *et al.*, 2002). Although less well characterized, the activity of S6K2 is also activated by mTOR (Park *et al.*, 2002).

However, the recent discovery that the PI3K/PKB/TSC/Rheb pathway lies upstream of mTOR highlights the complexity of mTOR regulation and indicates that PI3K-regulated growth factor signalling pathways are indeed involved in crosstalk with the nutrient sensing function of mTOR (Gao and Pan, 2001;Gao *et al.*, 2002;Inoki *et al.*, 2002;Potter *et al.*, 2002;Manning *et al.*, 2002).

1.2.5 Phosphorylation of S6K

S6K is fully activated by phosphorylation at multiple Ser/Thr residues mediated by several converging pathways (Pullen *et al.*, 1998;Ruvinsky and Meyuhas, 2006). Mitogen and nutrient-mediated pathways are the main contributors of upstream kinases which coordinate by phosphorylation of a complex

conformational conversion of S6K1 from the inactive to a fully activated state. To date, more than 10 phosphorylation sites involved in the activation process of S6K1 have been identified. The activation process can be subdivided into three main stages, reflecting a gradual increase in kinase activity (Figure 1.5). Mitogen and nutrient induced phosphorylation of S6K1 occur in a hierarchical fashion, starting with a cluster of serines and threonines residues located at the C-terminal autoinhibitory domain (Ser411, Ser418, Thr421, Ser424, Ser429, and Thr447 in the case of S6K1 II) (Figure 1.4). It has been proposed that in inactive conformation, the C-terminal autoinhibitory domain directly interacts with the kinase catalytic domain, blocking its ability to bind the substrate (Banerjee *et al.*, 1990). This interaction is believed to be strengthened by the association involving the acidic N-terminus and the basic region near the C-terminus. Phosphorylation of the C-terminal sites probably releases the autoinhibitory domain from the catalytic domain, thus priming the kinase for activation. It is most likely that these sites are phosphorylated by proline-directed kinases, as all of them contain proline in the +1 position and a hydrophobic amino acid in the -2 position (YXS/TP, where Y is a hydrophobic residue). Studies from numerous laboratories have implicated several proline-directed kinases, including SAPK (stress activated protein kinase), MAPK or ERK1/2 and cdc2 (cyclin-dependent kinase 2) (Mukhopadhyay *et al.*, 1992;Papst *et al.*, 1998). Mutational studies and PP2A treatment/reactivation experiments clearly indicated that phosphorylation of the pseudosubstrate domain is necessary, but not sufficient for full activation of S6K1 (Dennis *et al.*, 1998). These findings stimulated the search for other mitogen induced phosphorylation sites located in domains other than those which are autoinhibitory. As a result, additional phosphorylation sites have been identified in the kinase (Thr229) and kinase-extension domains (Ser371, Ser404 and Thr389). Phosphorylation of Thr389 was shown in numerous studies to be the second regulatory event mediating further conformational changes and partial activation of S6K1. This site is located in the kinase extension domain and is surrounded hydrophobic amino acids, which form a conserved sequence (F-X-X-F/Y-S/T-F/Y) found in other AGC kinases (Alessi *et al.*, 1998;Pullen *et al.*, 1998). Taking into account its hydrophobic nature, it is referred to in the

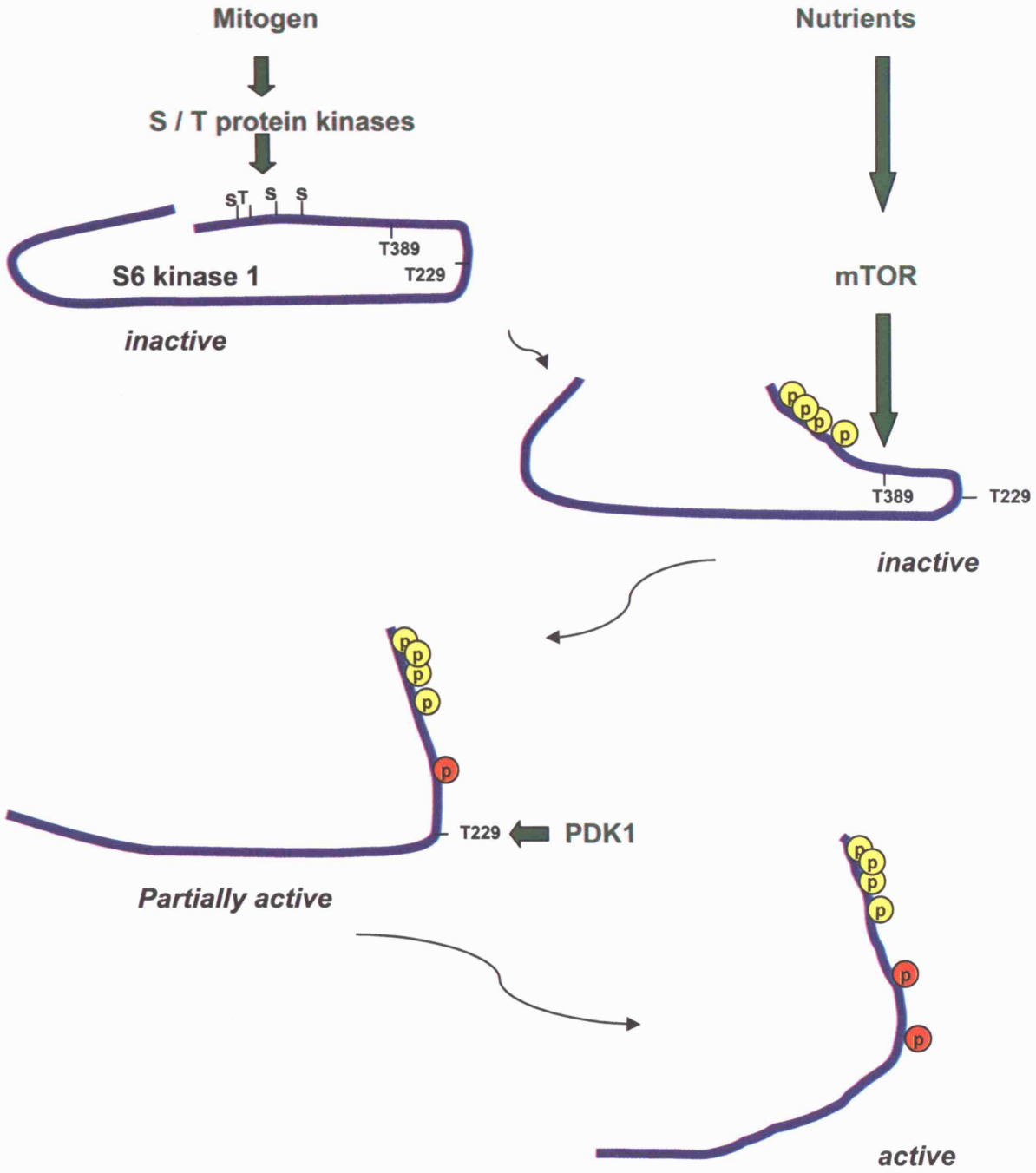


Fig. 1.5 A proposed model of S6K activation.

Multiple phosphorylation of S6K results in conformational changes which allow the access of its substrates to the catalytic domain. At the first stage, phosphorylation of four key C-terminal sites (Ser411, Ser418, Thr421, and Ser424 in the case of S6K1 II) opens the kinase domain for further phosphorylations. The mTOR phosphorylates S6K1 II at Thr389 resulting in partial activation of the kinase. The full activation of S6K1 II is completed by the phosphorylation on Thr229 carried out by PDK1.

literature as a hydrophobic motif. It has been demonstrated that phosphorylation of AGC kinases, including S6K, within the hydrophobic motif creates a high affinity binding platform for PDK1 (Biondi *et al.*, 2001).

A number of kinases have been implicated in directing phosphorylation of S6K1 at Thr389. These include PDK1 and NIMA-related kinases NEK6 and NEK7 (Balendran *et al.*, 1999b;Belham *et al.*, 2001). In addition, autophosphorylation of Thr389 has been also proposed by Blenis' laboratory (Romanelli *et al.*, 2002). Since Thr389 phosphorylation is sensitive to rapamycin, the involvement of mTOR has been also investigated. In fact, two separate laboratories both found that mTOR is capable of phosphorylating Thr389 and activating S6K1 *in vitro* (Burnett *et al.*, 1998a;Isotani *et al.*, 1999). The involvement of mTOR in Thr389 phosphorylation has been reinforced by the publication of a recent paper from Sabatini's laboratory, which details that mTOR phosphorylated an equivalent site (Ser473) in PKB/Akt (Sarbassov *et al.*, 2005b). However, the identity of the physiological Thr389 kinase remains uncertain.

Phosphorylation of S6K at the site located in the activation loop completes the activation process. The site has been identified as Thr229. The identity of the kinase responsible for its phosphorylation has been determined without controversy. PDK1 was found to phosphorylate Thr229 *in vitro* and *in vivo* (Alessi *et al.*, 1998;Pullen *et al.*, 1998). It is important to note, that other AGC kinases were shown to be phosphorylated by PDK1 at homologous sites in the activation loop. Bioinformatic analysis showed that hydrophobic motif and activation loop phosphorylation sites are highly conserved not only in S6K1 and S6K2, but also in other members of AGC family. The hierarchy of Thr389 and Thr229 phosphorylation has been clarified by mutational studies and their sensitivity to rapamycin. It was demonstrated that Thr229 phosphorylation is inhibited by treatment with rapamycin whereas PDK1 is not, implying indirect regulation of Thr229 phosphorylation by mTOR. Based on these data, it was proposed that rapamycin inhibits mTOR's ability to phosphorylate Thr389, thus preventing PDK1 from binding to the hydrophobic motif of S6K1 and

phosphorylating Thr229. In support of this idea, a T389E substitution elevates Thr229 phosphorylation, whereas T389A blocks it. Mutational analysis of the Thr229 site indicated that threonine to alanine substitution ablates S6K1 activity, but does not affect Thr389 phosphorylation (Pearson *et al.*, 1995). Crystal structure analysis of PKB/Akt kinase domain indicated that phosphorylation of the activation loop site Thr308 (Thr229 in S6K1) stabilises the kinase in a conformation that facilitates substrate binding (Yang *et al.*, 2002). Phosphorylation of the Thr229 and Thr389 sites closely correlates with mitogen and nutrient-induced activation of S6K1, and is sensitive to rapamycin and wortmannin treatment; therefore the phosphorylation status on these sites is often used as a gauge of S6K activity.

The N-terminal regulatory region was recently found to be crucial in mediating the mTOR activation signal to S6K1. Schalm and colleagues identified an mTOR signalling motif (TOS) at the extreme N-terminus of S6K1 and S6K2 (Schalm and Blenis, 2002). Similar motifs have also been located in 4E-BP1, another well studied physiological substrate of mTOR. Site-directed mutagenesis and deletion studies have provided evidence that TOS is a specific recognition motif for Raptor, a novel mTOR binding partner (Sabatini *et al.*, 1994;Hara *et al.*, 2002). Therefore, the association between mTOR and S6K which leads to Thr389 phosphorylation is arbitrated by a scaffold-like protein, Raptor.

Calcium mediated signalling has recently been implicated in priming S6K for activation (Hannan *et al.*, 2003). Calcium-driven regulation of S6K activity is possibly coordinated via the N-terminal region, since its deletion leaves S6K insensitive to calcium.

1.2.6 Upstream regulators of S6K

The signalling pathways and the major players involved in the activation of S6Ks are schematically presented in Figure 1.6.

1.2.6.1 PDK1

Phosphoinositide-dependent protein kinase 1 (PDK1) is a member of the AGC group of protein kinases. In the last decade, PDK1 has received attention because it was found to be responsible for the activation loop phosphorylation of several AGC protein kinases (Stokoe *et al.*, 1997;Alessi *et al.*, 1997b;Pullen *et al.*, 1998;Dutil *et al.*, 1998;Nilsen *et al.*, 2004). Extensive biochemical studies have clearly demonstrated that PDK1 is the upstream kinase for PKB/Akt, with phosphorylation at T308 in the activation loop of PKB/Akt. Following on the heels of the discovery that PDK1 is the PKB/Akt upstream kinase, came the observation that PDK1 also phosphorylates a number of other kinases, including S6K and protein kinase C (PKC).

PDK1 activates local substrate kinases by two mechanisms, direct or indirect. PKB/Akt and the atypical PKC ζ, for instance, are directly activated by PDK1 because phosphorylation at their activation loop serves as a "on/off" switch for catalytic activity. Once phosphorylated by PDK1, these kinases are directly activated. In contrast, phosphorylation of conventional PKC isozymes at the activation loop does not result in activation but rather "primes" PKC for subsequent activation (Dutil *et al.*, 1998).

Initial findings that PDK1 has a high basal level of activity, even in unstimulated cells, led to the notion that it is constitutively active, and that its activity is not critically regulated. However, recent studies have clearly demonstrated that the function of PDK1 is under tight control, with phosphorylation depending on

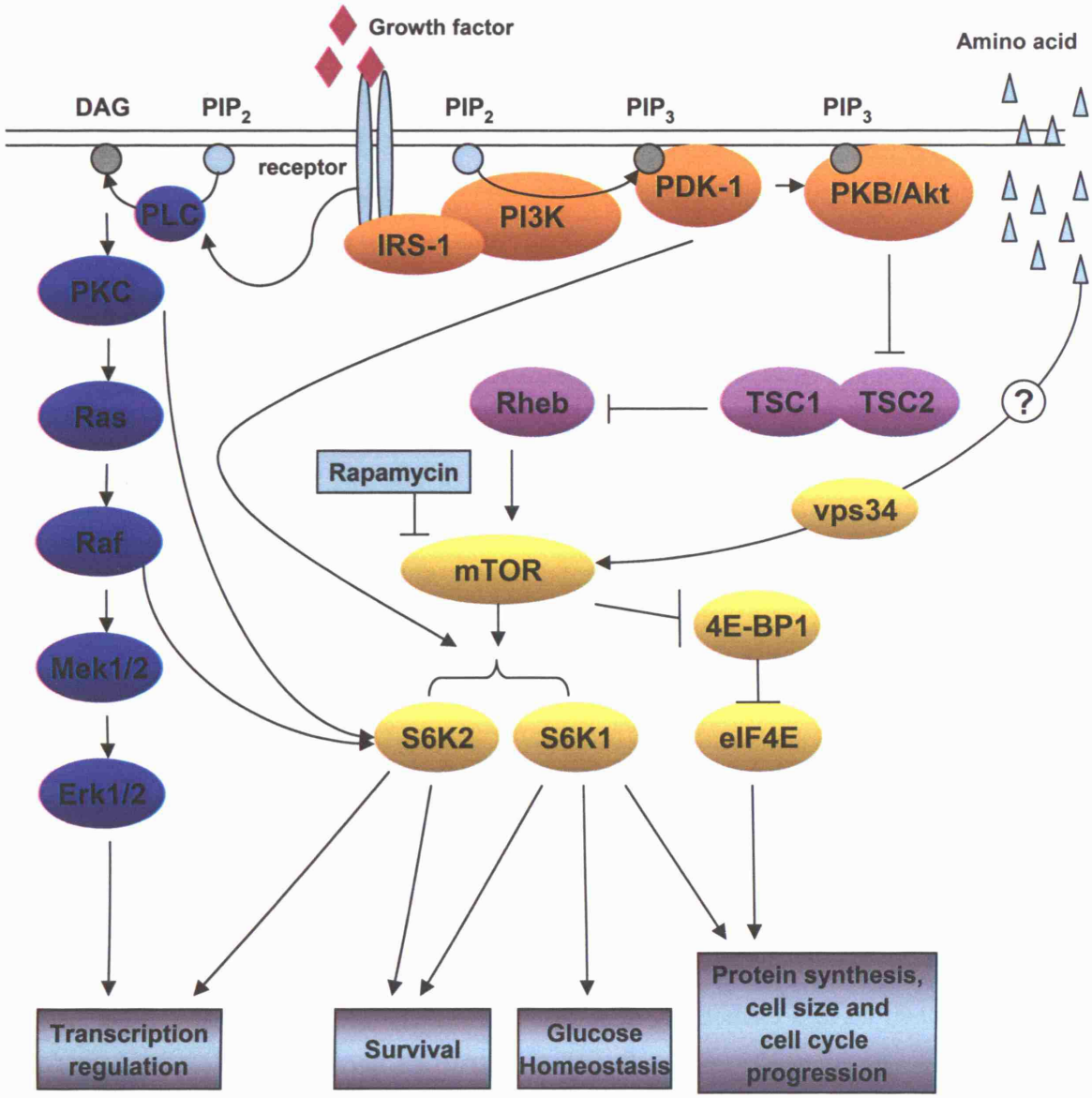


Fig. 1.6 The upstream networks of S6Ks.

The main upstream regulator of S6Ks, mTOR, is the central effector of growth factor and amino acid signals. The cascade of events starts by activation of the receptor tyrosine kinases through ligand binding, triggering the PI3K and the MAP signalling pathways, which consequently activate elements in the mTOR pathway and result in the involvement of several cellular regulations, such as transcription and translation, survival, glucose homeostasis, protein synthesis, cell size control, and cell cycle progression. Beside lying downsteam of signalling cascades activated by growth factors, the mTOR pathway also detects the cellular level of amino acid. The exact mechanism of the amino acid activation on mTOR pathway is however remained unclear. S6Ks are activated by direct phosphorylation, involving PDK1 and mTOR. In addition, the MAPK pathway also contributes to S6Ks activation. PLC, phospholipase C; PIP_2, phosphotidylinositol-4,5-diphosphate; PIP_3, phosphotidylinositol-3,4,5-triphosphate; DAG, diacylglycerol..

substrate conformation and subcellular location. The phosphorylation of PKB/Akt mediated by PDK1 is regulated by the conformation of PKB/Akt. The engagement of the PH domain on the membrane by binding PIP_3 or PIP_2 relieves autoinhibition of the active site allowing PDK1 to access Thr308 on the activation loop (Stokoe *et al.*, 1997). Consistent with this observation, an PKB/Akt mutant lacking the PH domain no longer requires PIP3 for PDK1-mediated phosphorylation *in vitro*, and so is constitutively phosphorylated and thus active in cells (Stokoe *et al.*, 1997;Filippa *et al.*, 2000). As a result, the PH domain masks the activation loop site and its release is required for PDK1 phosphorylation. Similarly, access of PDK1 to the activation loop of PKC is conformationally regulated. In this case, the autoinhibitory pseudosubstrate sequence of PKC must be removed from the binding cavity in order for PDK1 to phosphorylate PKC.

On the other hand, the binding of PIP3 to the PH domain of PKB/Akt and PDK1 co-localizes both proteins at the plasma membrane. This co-localization enables PDK1 to phosphorylate PKB/Akt in a phosphoinositide-dependent manner (Vanhaesebroeck and Alessi, 2000). Studies by Filippa and colleagues have also shown that PDK1 effectively recruits PKB/Akt to the plasma membrane in simulated cells: mutants of PDK1 deleted in its PH domain prevent translocation of PKB/Akt, whereas an PKB/Akt PH domain mutant efficiently translocates in the presence of intact PDK1 (Filippa *et al.*, 2000).

Phosphorylation of S6K by PDK1 also requires prior phosphorylation on its autoinhibitory sequence in order to expose the activation loop. Thus, substrate conformation is a major determinant in allowing PDK1 phosphorylation to occur. However, unlike PKB/Akt, which is co-localized with PDK1 at the plasma membrane through the PH domain, S6K interacts directly with PDK1 through a region that is at the C-terminal end of the catalytic domain within the conserved Phe-Xaa-Xaa-Phe hydrophobic motif (HM). In some AGC kinases, the HM contains a contiguous phosphorylation site (Phe-Xaa-Xaa-Phe-[Ser/Thr]-Tyr), in which the serine or threonine residues correspond to the HM phosphorylation site,

and interaction with PDK1 appears to be triggered by prior phosphorylation of this motif. HM phosphorylation is crucial for the interaction of PDK1 with substrates such as S6K, SGK, RSK, and CISK (Balendran *et al.*, 1999b;Frodin *et al.*, 2000;Biondi *et al.*, 2001;Frodin *et al.*, 2002;Nilsen *et al.*, 2004). The requirement of HM phosphorylation in S6K could explain why, upon stimulation of the cell, a temporal difference is evident between PKB/Akt phosphorylation (which is more rapid) and the phosphorylation of S6K by PDK1.

1.2.6.2 PKB/Akt

PKB/Akt is a serine/threonine kinase, belonging to the AGC superfamily of protein kinases which share structural similarity within the catalytic domain and have similar mechanisms of activation. The initial identification of PKB/Akt was characterized by three independent groups, based on its homology to protein kinase (PKA) (Coffer and Woodgett, 1991) and C (PKC) (Jones *et al.*, 1991b) or as the cellular homolog to the retroviral oncogene *v-akt* (Bellacosa *et al.*, 1991). Its close relation to PKA and PKC led to it being named PKB by the authors of one of these studies. To date, three members of the family have been isolated and these are now referred to as PKBα/Akt1 (Jones *et al.*, 1991a), PKBβ/Akt2 (Cheng *et al.*, 1992), and PKBγ/Akt3 (Brodbeck *et al.*, 1999), located at chromosomes 14q32, 19q13, and 1q44, respectively. The three isoforms exhibit greater than 80% identity at the amino acid level but are expressed differentially.

All three PKB/Akt isoforms consist of a conserved domain structure: an amino-terminal pleckstrin homology (PH) domain, a central catalytic domain, and a carboxyl-terminal regulatory domain containing the hydrophobic motif, a characteristic feature of AGC kinases. The N-terminal PH domain consists of approximately 100 amino acids. Recent detailed structural examination of PKB/Akt PH domains reveals similarity to PH domains found in other signalling molecules that bind 3-phosphoinositides (Lietzke *et al.*, 2000;Ferguson *et al.*,

2000). The PH domain interacts with membrane lipid products such as phosphatidylinositol-(3,4,5)-trisphosphoate (PIP_3) produced by phosphatidylinositol 3-kinase (PI3K). Biochemical analysis revealed that the PH domain of PKB/Akt binds to both PIP_2 and PIP_3 with similar affinity (James *et al.*, 1996;Frech *et al.*, 1997). The catalytic domain located in the central region of the molecule shares a high degree of similarity with other AGC kinases such as PKA, PKC, S6K, and p90RSK (Peterson and Schreiber, 1999). There is a conserved threonine residue (Thr308 in PKBα/Akt1) in the catalytic domain which, when phosphorylated, partially activates PKB/Akt (Alessi *et al.*, 1996a). A hydrophobic tail containing around 40 amino acids is located C-terminally to the catalytic domain. This region possesses the F-X-X-F/Y-S/T-Y/F motif (where X is any amino acid) that is characteristic of the AGC kinase family (Peterson and Schreiber, 1999). A second regulatory phosphorylation site (Ser473 in PKBα/Akt1) within this region has been reported and the C-terminal of PKB/Akt was shown to be critically important, because a deletion mutant of this motif completely halted enzymatic activity (Andjelkovic *et al.*, 1997).

Phosphorylation at sites Thr308 in the activation loop of the catalytic domain and Ser473 in the C-terminal regulatory domain occurs in response to growth factors and other extracellular stimuli. It is essential for maximal PKB/Akt activation that both sites are phosphorylated, while phosphorylation singly on either site only partially activates kinase activity (Alessi *et al.*, 1996a). The mechanism of PKB/Akt activation can be divided into two steps: a) membrane translocation step and b) phosphorylation step. The first step is mediated in a PI3K- and PH domain-dependent manner. Binding to the phospholipid produced by PI3K through PH domain of PKB/Akt recruits the kinase to the plasma membrane, possibly resulting in a conformational change, which then allows subsequent phosphorylation by the phosphoinositide-depedent kinase-1 (PDK1).

It is generally accepted that PDK1 phosphorylates PKBα/Akt1 at Thr308. For PKB/Akt, co-localization with PDK1 is necessary for phosphorylation on Thr308 (Andjelkovic *et al.*, 1997;Anderson *et al.*, 1998). Although membrane

recruitment of the two proteins gives rise to PDK1 to phosphorylate PKB/Akt, binding of the PKB/Akt PH domain to phosphoinositide could result in a conformation change which may unveil the site Thr308 to PDK1 (Stokoe *et al.*, 1997;Alessi *et al.*, 1997a). A PKB/Akt deletion mutant of the PH domain, or a peptide substrate corresponding to the activation loop of PKB/Akt containing a PDK1 phosphorylation site, renders the substrates to be phosphorylated by PDK1 in PIP_3-independent manner (Biondi *et al.*, 2000).

The mechanism by which Ser473 phosphorylation is mediated remains the subject of controversy. PDK1 is a possible candidate responsible for this site, as Ser473 phosphorylation is dependent on PI3K, as well as Thr308 (Balendran *et al.*, 1999a). Several groups reported that PKB/Akt itself could autophosphorylate Ser473 under certain conditions (Toker and Newton, 2000;Laine *et al.*, 2000). Other findings suggest that Ser473 is modified by a distinct kinase such as PDK-2 (Hill *et al.*, 2001). The integrin-linked kinase (ILK) was also shown to phosphorylate Ser473. A more recent study showed that ILK could phosphorylate PKB/Akt on Ser473, and that the kinase activity of ILK was essential for Ser473 phosphorylation in cells, which was blocked by a novel ILK-specific inhibitor (Persad *et al.*, 2001). These data strongly suggest that ILK plays a role in the activation process, but whether it phosphorylates PKB/Akt directly is a question still to be answered. Recently, a study from Sabatini's group provided undisputed evidence for mTOR as the kinase which specifically phosphorylates PKB/Akt at Ser473 *in vitro* and *in vivo* (Sarbassov *et al.*, 2005b). Recruiting a variety of biochemical, mutational, and siRNA approaches, they showed that mTOR kinase and its associated protein rictor are necessary for Ser473 phosphorylation in PKB/Akt. In addition, the researchers showed that the phosphorylation of PKB/Akt by rictor-mTOR complex facilitated Thr308 phosphorylation by PDK1.

In addition to Ser/Thr phosphorylation, several tyrosine residues, such as Y315 and Y326, have been reported as important factors in PKB/Akt activity (Chen *et al.*, 2001). This report suggested that these modifications are dependent on Src

family tyrosine kinases. While phosphorylation is the main mechanism to activate PKB/Akt, the dephosphorylation pathway is believed to be an inhibitory signal that suppresses PKB/Akt activity. Using phosphatase inhibitor, such as okadaic acid, this hypothesis was proved by Andjelkovic and colleague in 1996 (Andjelkovic *et al.*, 1996).

PKB/Akt directs many cellular functions by interacting with a number of substrates, such as BAD (a member of the Bcl-2 family of proteins that binds to Bcl-2 or Bxl-X and inhibits their anti-apoptotic potential) and Caspase-9 (an initiator and effecter of apoptosis) in the anti-apoptosis pathway (del Peso *et al.*, 1997;Datta *et al.*, 1997;Cardone *et al.*, 1998) and Foxo Forkhead (a family of transcription factors), NF-kB (transcription factor nuclear factor-kB, a key regulator of the immune response), CREB (Cyclic AMP-response element binding protein), and YAP (Yes-associated protein) in transcription regulation (Du and Montminy, 1998;Kane *et al.*, 1999;Burgering and Medema, 2003;Basu *et al.*, 2003).

mTOR/S6K is another downstream effector of PKB/Akt, through which it controls cell growth and size. Initial evidence appeared in studies which demonstrated that mTOR is a direct substrate of PKB/Akt (Nave *et al.*, 1999;Sekulic *et al.*, 2000). The regulation of mTOR/S6K by PKB/Akt was not clear until it was realised that TSC1/2 and Rheb are participants in the link between the two pathways, which will be discussed later in this chapter. The PKB/Akt-mTOR pathway also regulates the transcription level of cyclin D by a mechanism that appears to involve CREB (Muise-Helmericks *et al.*, 1998;D'Amico *et al.*, 2000). Evidence also showed that mTOR pathway is involved in oncogenic signalling by PKB/Akt (Aoki *et al.*, 2001).

PKB/Akt may also be activated in a PI3K-independent manner, mostly mediated through PKA (Sable *et al.*, 1997;Filippa *et al.*, 1999). The PH domain of PKB/Akt is not required for this activation and phosphorylation of Ser473 is not necessary for the PKA induced activation, while the phosphorylation of Thr308 is

required. Other activation mechanisms independent of PI3K have also been reported by different groups. However, the significance of these findings remains to be determined.

1.2.6.3 mTOR

The TOR (target of rapamycin) genes were originally identified in yeasts as the target of rapamycin (Heitman *et al.*, 1991). The structurally and functionally conserved mammalian counterpart of yeast TOR, was later discovered and termed mTOR (Brown *et al.*, 1994;Chiu *et al.*, 1994;Sabatini *et al.*, 1994). There are two TOR genes in yeast, which encode highly related proteins (Tor1p and Tor2p) of approximately 290 kDa. Both proteins have been identified and characterized as catalytic cores in large protein complexes called TOR complex 1 (TORC1) and TOR complex 2 (TORC2). Both Tor1p and Tor2p are found in TORC1, which mainly regulates cell growth in a rapamycin-sensitive manner; whereas TORC2 consists solely of Tor2p and regulates cytoskeletal organization in a rapamycin–insensitive manner. In mammalian cells, mTOR also exists in two distinct forms, termed mTORC1 and mTORC2 complexes (Hara *et al.*, 2002;Kim *et al.*, 2002;Loewith *et al.*, 2002;Sarbassov *et al.*, 2004).

The TOR is a Ser/Thr protein kinase (~290 kDa) containing more than 2500 amino acids (Brown *et al.*, 1994). It is highly conserved from yeast to mammals. mTOR shares around 45% identity with the S. Cerevisiae Tor1 and 2 proteins, and 56% identity with dTOR (Brown *et al.*, 1994;Sabatini *et al.*, 1994;Sabers *et al.*, 1995;Zhang *et al.*, 2000;Oldham *et al.*, 2000). The human, mouse, and rat mTOR proteins share approximately 95% identity at the amino acid level. A number of mTOR binding partners have recently been identified: a) the scaffold substrate-presenting protein raptor (the yeast homologue of which is Kog1), b) the protein GβL (the yeast homologue of which is Lst8), c) the scaffold substrate-presenting protein rictor (the yeast homologue of which is Avo3), d) the small GTP-binding protein Rheb (the yeast homologue of which is LSt8), the

adaptor-like protein mSin1 (the yeast homologue of which is Avo1), and e) the protein PRAS, a negative regulator of mTOR signalling (Kim *et al.*, 2002;Loewith *et al.*, 2002;Kim *et al.*, 2003).

Studies from various laboratories have indicated that mTOR exists in two functionally distinct complexes, termed mTOR complex 1 (mTORC1) and mTORC2. mTORC1 contains the core components mTOR, raptor and mLST8/GβL, and is sensitive to rapamycin. mTORC2 is believed to be rapamycin-insensitive and contains mTOR, rictor and mLST8/GβL (Sarbassov *et al.*, 2005a;Corradetti and Guan, 2006;Tsang *et al.*, 2007). As TORC2 is not a target of rapamycin and is not linked to the activation of S6K pathway, it will not be considered further here.

The general domain structure of mTOR, as shown in Figure 1.7, is similar to that of yeast TOR proteins. Close to the mTOR C-terminus lies a domain displaying sequence similarity to the kinase domain of the lipid kinase, PI3K. As a result, mTOR is classified as a member of PI 3-kinase-related kinases (PIKK), which also includes MEC1, TEL1, RAD3, MEI41, DNA-PK, ATM, ATR, and TRAPP (Brown *et al.*, 1994;Jacinto and Hall, 2003;Miranda-Saavedra and Barton, 2007). However, despite the significant sequence homology to lipid kinases, there is no direct evidence that mTOR phosphorylates lipids.

The extreme C-terminus region of mTOR contains a domain, termed the FATC domain, conserved in other PIKK members (Alarcon *et al.*, 1999;Bosotti *et al.*, 2000). Deletion of a single amino acid from the FATC domain, or the addition of an epitope tag to the C terminus, effectively eliminates mTOR activity (Takahashi *et al.*, 2000). The reason mutations in this region have such a major effect on mTOR function is unknown.

A domain called FRB (FKBP12/rapamycin binding domain) is located at the N-terminal side of the kinase domain. This region is the site through which rapamycin inhibits mTOR function (Chen *et al.*, 1995;Choi *et al.*, 1996). The

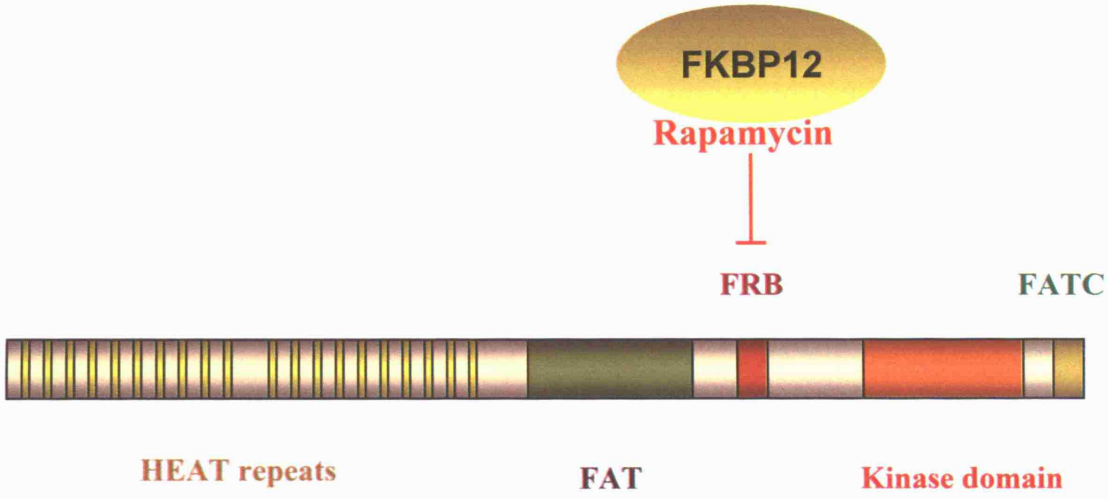


Fig. 1.7 Domain organization of mTOR.

The general domain structure of mTOR is presented in this figure. Close to the mTOR C-terminus lies its kinase domain, displaying sequence similarity to that of PI3K. As a result, mTOR is classified as a member of PIKK family. However, despite the significant sequence homology to lipid kinases, there is no direct evidence that mTOR phosphorylates lipids. The extreme C-terminus region of mTOR contains a domain, termed the FATC domain, conserved in other PIKK members. A region called FRB (FKBP12/rapamycin binding domain) is located at the N-terminal side of the kinase domain. This region is the site through which rapamycin inhibits mTOR function. At the N-terminal domain of the FRB sits a domain which is conserved in other members of PIKK family, named the FAT domain. Like FATC, its role in the function of PIKKs is unclear. The remainder of the mTOR molecule, from the N-terminus to the FAT region, comprises up to twenty copies of HEAT motif (Huntingtin-elongation factor 1A-protein phosphatase 2A subunit-TOR repeats). These 39-amino acid repeats most likely form a pair of antiparallel α-helices, stacking with other HEAT repeats to form a surface that is capable of multiple protein-protein interactions. To inhibit mTOR, rapamycin binds first to FKBP12, resulting a complex association with the FRB domain.

Ser2035 located within the relatively hydrophobic rapamycin-binding pocket is crucial for mTOR and rapamycin interaction, as replacement of Ser2035 with any residue larger than Ala blocks binding of FKBP12-rapamycin, thus generating a rapamycin-resistant form of mTOR (Chen *et al.*, 1995;McMahon *et al.*, 2002). The FRB domain is absent from DNA-PK, ATM or ATR, and consequently these kinases are not affected by rapamycin.

At the N-terminal domain of the FRB sits a domain which is conserved in other PIKKs, named the FAT domain. Like the FATC domain, its role in the function of PIKKs is unclear. The FAT domain has also been termed the toxic-effector domain, because its overexpression in yeast leads to a G1 cell cycle arrest (Takahashi *et al.*, 2000). This effect was hypothesized to be due to sequestration of necessary interacting proteins away from TOR.

The remainder of the mTOR molecule, from the N-terminus to the FAT region, comprises up to twenty copies of HEAT motif (Huntingtin-elongation factor 1A-protien phosphatase 2A subunit-TOR repeats) (Andrade and Bork, 1995). These 39 amino acid repeats most likely form a pair of antiparallel α-helices, stacking with other HEAT repeats to form a surface that is capable of multiple protein-protein interactions (Groves *et al.*, 1999).

The subcellular distribution of TOR is not well defined. In yeast, two independent groups have reported the localization of TOR proteins, which appear to be associated with membranes (Kunz *et al.*, 2000;Wedaman *et al.*, 2003). In contrast, there are huge discrepancies in the reported locations of mTOR within mammalian cells. Some findings are consistent in part with the membrane association of the yeast TOR (Withers *et al.*, 1997;Drenan *et al.*, 2004), whereas other experiments suggested that most of the protein associates with mitochondria (Desai *et al.*, 2002). In addition, confocal immunofluorescence microscopy used in murine myoblasts, human fibroblasts, and several malignant cell lines showed that most mTOR in these cells appeared to be nuclear (Zhang *et al.*, 2002). Additional studies are clearly required to fully resolve this issue.

Rapamycin and its derivatives are the subject of many clinical trials and are already used in clinics to inhibit host rejection of transplanted organs and prevent the occlusion of coronary arteries after angioplasty (Odorico and Sollinger, 2002;Garza *et al.*, 2002;Huang and Houghton, 2003). In addition to its major value as a clinical drug, rapamycin is also an experimental tool for the investigation of TOR functions in various experimental settings. Using rapamycin has been a valuable strategy for the identification of the downstream targets of TOR proteins, and has revealed that mTOR plays a role in the regulation of a variety of cellular functions and processes. The most well characterized downstream targets are the translational regulators, S6K and 4E-BP1. mTOR enhances S6K activity and suppresses 4E-BP1, giving the cell the advantage of growth and proliferation, by phosphorylating each of the proteins. (Harris and Lawrence, Jr., 2003;De Virgilio and Loewith, 2006;Wullschleger *et al.*, 2006). As well as S6K and 4E-BP1, numerous other proteins have been reported as targets for TOR signalling: including eukaryotic elongation factor 2 (eEF2) kinase (Browne and Proud, 2002) and proteins involved in the control of transcription, such as HIF1α, STAT3, Rb and components of signalling pathways, PKC isoforms δ and ε, protein phosphatase 2A and PKB/Akt (Harris and Lawrence, Jr., 2003). The elucidation of the mechanisms involved in their control represents a major aim for further work in this area.

1.2.7 Negative control of S6K signalling

1.2.7.1 Protein phosphatases

The phosphorylation state of proteins is regulated by both kinases and phosphatases. Therefore, the reversible phosphorylation-dephosphorylation of target proteins can be viewed as a binary switch in which kinases and phosphatases push the target into the "on" or "off" state. In contrast to kinases,

phosphatases remove phosphate groups from specific residues, resulting in either activation or inactivation of target proteins. There are two major classes of protein phosphatases: those which specifically dephosphorylate serine or threonine (Ser/Thr) residues and those which remove the phosphate from tyrosine (Tyr). It is important to note that some phosphatases have dual specificity for dephosphorylating both Ser/Thr and Tyr residues. The family of protein Ser/Thr phosphatases can be divided into several classes, class 1 (PP1), class 2A (PP2A), class 2B (PP2B), and class 2C (PP2C), according to their substrate preference and sequence homology. It has been observed that PP2A coimmunoprecipitates with S6K1 (Begum and Ragolia, 1996;Westphal *et al.*, 1999), which is though to be the mechanism by which S6K1 is inactivated by dephosphorylation at certain residues, crucial for S6K1 activity.

In addition to phosphorylation of S6K at Ser389, mTOR may also activate S6K via the inhibition of protein serine/threonine phosphatase. Evidence supporting the phosphatase model includes the well-documented regulation of a PP2A-like phosphatase in budding yeast by rapamycin (Lorberg and Hall, 2004), and the rapid loss of S6K1 multi-site phosphorylation upon rapamycin addition to cells (Chung *et al.*, 1992). In mammalian cells, rapamycin has been reported to activate PP2A, specifically in Jurkat cells (Peterson *et al.*, 1999), and to prevent the inactivation of PP2A by insulin in rat skeletal muscle cells (Begum and Ragolia, 1996). The effects of rapamycin suggest that mTOR controls PP2A activity.

1.2.7.2 PTEN phosphatase, indirect inhibitor of S6K

A variety of biochemical, mutational and genetic studies identified PTEN as a negative regulator of S6K signalling (Cortot *et al.*, 2006). Several downregulation mechanisms ensure that PIP_3 formation is transient; these include the SH2-containing inositol D5-phosphatases (SHIP), which transform PIP_3 into PIP_2, and the D3-phosphatase PTEN (phosphatase and tensin homologue deleted

on chromosome ten). PTEN reduces both PIP_3 and PIP_2 levels, although details as to how this action is regulated remain unclear (Maehama and Dixon, 1998;Parsons, 2004).

In many respects, the major breakthrough in understanding PI3K signalling in normal cells and its deregulation in cancer, was the realization that the tumor suppressor PTEN was a PtdIns(3,4,5)P_3 3-phosphatase. PTEN is a dual-specificity enzyme, which possesses lipid and protein phosphatase activities, and was originally identified as a tumor suppressor gene. The main physiological substrate of PTEN lipid phosphatase is PI(3,4,5)P_3, the product of PI3K activity. PTEN dephosphorylates PI(3,4,5)P_3 at 3'-inositol position and in this way, acts as a negative regulator for PI3K-induced pathway (Wu *et al.*, 1998;Maehama and Dixon, 1998). Mutations which inactivate PTEN lipid phosphatase activity, result in constitutively active PKB/Akt and uncontrolled PI3K signalling (Haas-Kogan *et al.*, 1998). Studies on *PTEN* $^{+/-}$ mice demonstrated an increase in the incidence of tumor formation in the colon, testes, and thyroid (Di Cristofano *et al.*, 1998;Stambolic *et al.*, 1998;Myers *et al.*, 1998). Loss-of-function mutations of the tumor suppressor PTEN in human cancers occur with a frequency equaling that of the tumor suppressor p53 (Cantley and Neel, 1999;Simpson and Parsons, 2001). Moreover, overexpression of *PTEN* in breast cancer cell lines suggested that it acts as a tumor suppressor by inhibiting cell growth and enhancing cellular sensitivity for apoptosis and anoikis, a particular type of apoptosis induced in epithelial cells due to alterations in integrin-extracellular matrix interaction (Weng *et al.*, 1999;Lu *et al.*, 1999).

The negative regulation of mTOR/S6K signalling by tumor suppressor PTEN is undisputedly demonstrated in various experimental setups and models. Therefore, cells lacking or expressing lipid phosphatase-dead mutants of PTEN should be sensitive to mTOR inhibitors. Indeed, it has been found that transformed cells of *PTEN* $^{+/-}$ mice have elevated levels of phosphorylated Akt and activated S6K, associated with an increase in proliferation (Podsypanina *et al.*, 2001). The inactivation of mTOR by rapamycin resulted in reduced neoplastic

proliferation, tumor size and S6K activity, but did not affect the status of Akt.

In addition, Neshat and colleagues provided further evidence on the enhanced sensitivity of tumors lacking PTEN phosphatase activity to mTOR inhibitors (Neshat *et al.*, 2001). These results provide rationale for testing FRAP/mTOR inhibitors in PTEN null human cancers. The two original studies were followed by numerous publications describing the efficacy of rapamycin and its homologues in inhibiting the growth of tumor cells, lacking PTEN function (Shi *et al.*, 2002;Gera *et al.*, 2004;Rubio-Viqueira and Hidalgo, 2006;Hernando *et al.*, 2007;Tanaka *et al.*, 2007;Nathan *et al.*, 2007). Furthermore, the inhibition of mTOR signalling resulted in reversed doxorubicin resistance, conferred by PTEN status in prostate cancer cells (Grunwald *et al.*, 2002).

These data suggest that S6K and possibly other targets of mTOR contribute significantly to tumor development and that inhibition of these proteins may be therapeutic for cancer patients with disregulated PI3K signalling involving tumor suppressor PTEN. It is important to note that anti-cancer activity of mTOR orthologs alone or in combination with known cytotoxic drugs is currently being tested in numerous clinical trials.

1.2.7.3 Tumor suppressor TSC1/2 complex as a negative regulator of S6K pathway

The recent identification of the tuberous sclerosis complex (TSC) gene products has lightened the regulatory link between PI3K and TOR pathway. Tuberous Sclerosis is an autosomal dominant disorder that affects approximately 1 in 6000 individuals (Leung and Robson, 2007). This disease is characterized by the widespread development of benign tumors termed harmatomas, frequently leading to skin rashes, seizures, and mental retardation. Tuberous Sclerosis is caused by mutations on one of two tumor suppressor genes: *TSC1*, encoding harmartin, or

TSC2, encoding tuberin (Leung and Robson, 2007). Harmartin and tuberin form a TSC1-TSC2 complex (van Slegtenhorst *et al.*, 1998;Nellist *et al.*, 1999), which genetic evidence suggests is essential for complete function of the two proteins (Tapon *et al.*, 2001;Potter *et al.*, 2001;Gao and Pan, 2001).

The first indication of the biochemical link between PI3K signalling and TSC1/2 complex came from genetic studies in *Drosophila*. Mutation of *Drosophila* PTEN is similar to that of TSC1/TSC2 (Potter *et al.*, 2001). Genetic epistasis analyses placed the *Drosophila* homologues of harmartin (dTSC1) and tuberin (dTSC2) in a pathway which antagonizes insulin signalling downstream of, or parallel to, the *Drosophila* PI3K pathway (Tapon *et al.*, 2001;Potter *et al.*, 2001;Gao and Pan, 2001). Moreover, PKB/Akt, a downstream effector of PI3K signalling, was found by three independent groups to phosphorylate tuberin in rats, *Drosophila*, and humans (Inoki *et al.*, 2002;Potter *et al.*, 2002;Manning *et al.*, 2002). It appears that PKB/Akt phosphorylates human tuberin on three positions (Ser939, Ser1130, and Thr1462), thereby providing a direct biochemical link from the PI3K pathway to the TSC complex.

Phosphorylation by PKB/Akt is generally accepted as an inhibitory mechanism of TSC complex on the mTOR pathway and subsequent cell growth (Inoki *et al.*, 2002;Potter *et al.*, 2002;Manning *et al.*, 2002). How phosphorylation on TSC complex affects its function, however, is still unclear. For example, one model suggested that PKB/Akt phosphorylation might decrease the stability of TSC complex, or disrupt the formation of the tuberin-harmartin complex (Inoki *et al.*, 2002;Potter *et al.*, 2002), while Manning and colleagues claim that no correlation between growth factor-induced phosphorylation of tuberin and the amount of associated harmartin was detected (Manning *et al.*, 2002). Beside TSC complex stability and tuberin-harmartin interaction, the changes of its subcellular localization (Potter *et al.*, 2002), the interaction of TSC complex with 14-3-3 (Nellist *et al.*, 2002;Li *et al.*, 2002;Liu *et al.*, 2002;Shumway *et al.*, 2003), and the effects on the GAP activity of tuberin are also possible mechanisms by which PKB/Akt inhibits TSC tumor suppressor activity.

Although the molecular function of the TSC complex has yet to be determined, there is now considerable evidence demonstrating a role in regulation of S6K. Studies in *Drosophila* suggested that dS6K is genetically epistatic to dTSC1 and dTSC2, and that the dTSC1-dTSC2 complex negatively regulates dS6K function in cell-size control (Tapon *et al.*, 2001;Potter *et al.*, 2001). Additionally, $TSC1^{-/-}$ mouse embryonic fibroblasts and $TSC2^{-/-}$ rat smooth muscle and epithelial cells exhibit constitutive activation of S6K1 (Kwiatkowski *et al.*, 2002;Goncharova *et al.*, 2002), further suggesting that the TSC complex somehow negatively regulates S6K. Furthermore, the use of siRNA and mouse knockouts showed that cells with a loss of harmartin or tuberin exhibit constitutive phosphorylation of two distinct downstream mTOR targets, S6K1 and 4E-BP1 (Inoki *et al.*, 2002;Kwiatkowski *et al.*, 2002), which require mTOR function. Reciprocally, overexpression of harmartin and tuberin together, but neither alone, blocks both the phosphorylation/activation of S6K1 and the phosphorylation/inhibition of 4E-BP1 (Inoki *et al.*, 2002;Tee *et al.*, 2002). Moreover, two independent groups found that a rapamycin-resistant mutant of S6K, which is activated in a TOR-independent manner, is also resistant to inhibition by overexpression of TSC complex. Furthermore, TSC1 and TSC2 were also found to physically associate with TOR (Gao *et al.*, 2002).

The mechanism by which TSC negatively regulates TOR/S6K signalling has remained elusive. However, recent studies demonstrated that a small GTPase, Rheb, mediates the phosphorylation and activation of mTOR and plays an essential role in the regulation of the two main downstream effectors of TOR, S6K and 4EBP1, while TSC has an inhibitory effect on Rheb, therefore repressing the TOR pathway (Garami *et al.*, 2003;Inoki *et al.*, 2003a). This finding suggests a role for Rheb as direct target of TSC complex in the regulation of TOR signalling. However, the method by which Rheb signals to TOR still needs further investigation.

The involvement of the AMP-activated protein kinase (AMPK) in the regulation TSC1/2 complex, and subsequently mTOR signalling, has recently been

uncovered. AMPK is regarded as an "energy sensor" because it binds to and is regulated by both AMP and ATP. The binding of AMP to AMPK allows it to be phosphorylated by upstream kinases, such as tumor suppressor LKB. Phosphorylation of AMPK by LKB is crucial in the regulation of its kinase activity (Hardie, 2005). In contrast, the binding of ATP prevents its activation. AMPK regulates a multitude of metabolic processes that cumulatively function to maintain cellular energy homeostasis and to regulate biosynthetic processes in response to extracellular cues. The AMPK signalling network is regulated by a number of tumor suppressor genes including LKB1, p53, TSC1 and TSC2 which direct the inhibition of growth factor/mitogen/nutrient signalling (Hardie, 2004). One downstream AMPK target that has been recently identified is the mammalian target of rapamycin (mTOR), a positive effector of cell growth and division.

Several laboratories have reported that activation of AMPK suppresses mTOR signalling in response to growth factors and nutrients(Bolster *et al.*, 2002;Shaw *et al.*, 2004;Kimball, 2006;Gleason *et al.*, 2007). This inhibitory effect is mediated by phosphorylation of TSC2 at Thr-1227 and Ser-1345. Phosphorylation of TSC2 at these sites, which are distinct from PKB and ERK phosphorylation sites, increases TSC2 GAP activity towards Rheb, subsequently leading to the inhibition of mTOR/S6K signalling and cell growth (Inoki *et al.*, 2003b;Corradetti *et al.*, 2004;Shaw *et al.*, 2004;Inoki *et al.*, 2006). Furthermore, AMPK was recently reported to phosphorylate mTOR at Thr-2446 which suppresses S6K1 activation by insulin (Cheng *et al.*, 2004b). Taken together, signal transduction via AMPK directly (mTOR phosphorylation) and indirectly (TSC2 phosphorylation) suppresses mTOR activity and its downstream signalling to affect cell growth and proliferation in response to energy status or nutrient availability.

1.3 Downstream signalling and physiological roles of S6Ks

Genetic studies employing mice and *Drosophila* melanogaster have provided new insights into the physiological function of S6K (Shima *et al.*, 1998;Montagne *et al.*, 1999;Pende *et al.*, 2004). The outcome of these studies confirmed the paramount importance of S6Ks on growth control, glucose and energy homeostasis. Discovery of new signalling networks mediated by the S6K pathway, and the identification of novel downstream targets, is required for better understanding of its function in cellular processes. So far, very few substrates for S6K1/2 have been identified. The ribosomal protein S6, a component of the 40S ribosome, is the main physiological substrate for S6K. Additionally, incompletely characterized S6K binding partners and substrates have been found, including the transcription factor CREM τ, the export factor CBP80, the apoptotic protein BAD, and eEF2 kinase (eEF2K), neurabin and SKAR (Figure 1.8) (de Groot *et al.*, 1994;Brennan *et al.*, 1999;Haruta *et al.*, 2000;Tremblay and Marette, 2001;Harada *et al.*, 2001;Wang *et al.*, 2001c;Raught *et al.*, 2004;Proud, 2004;Richardson *et al.*, 2004;Maquat, 2004;Holz and Blenis, 2005;Chiang and Abraham, 2005;Shahbazian *et al.*, 2006). Characterising the functional importance of these interactions/phosphorylations led to the realization of the involvement of S6K signalling in various cellular processes, such protein synthesis, cell survival, growth regulation and glucose homeostasis.

1.3.1 Regulation of translation

1.3.1.1 S6Ks and translation of 5'TOP RNAs

It was widely considered that S6K1-mediated phosphorylation of ribosomal S6 protein controls the translation of mRNAs containing an oligopyrimidine tract

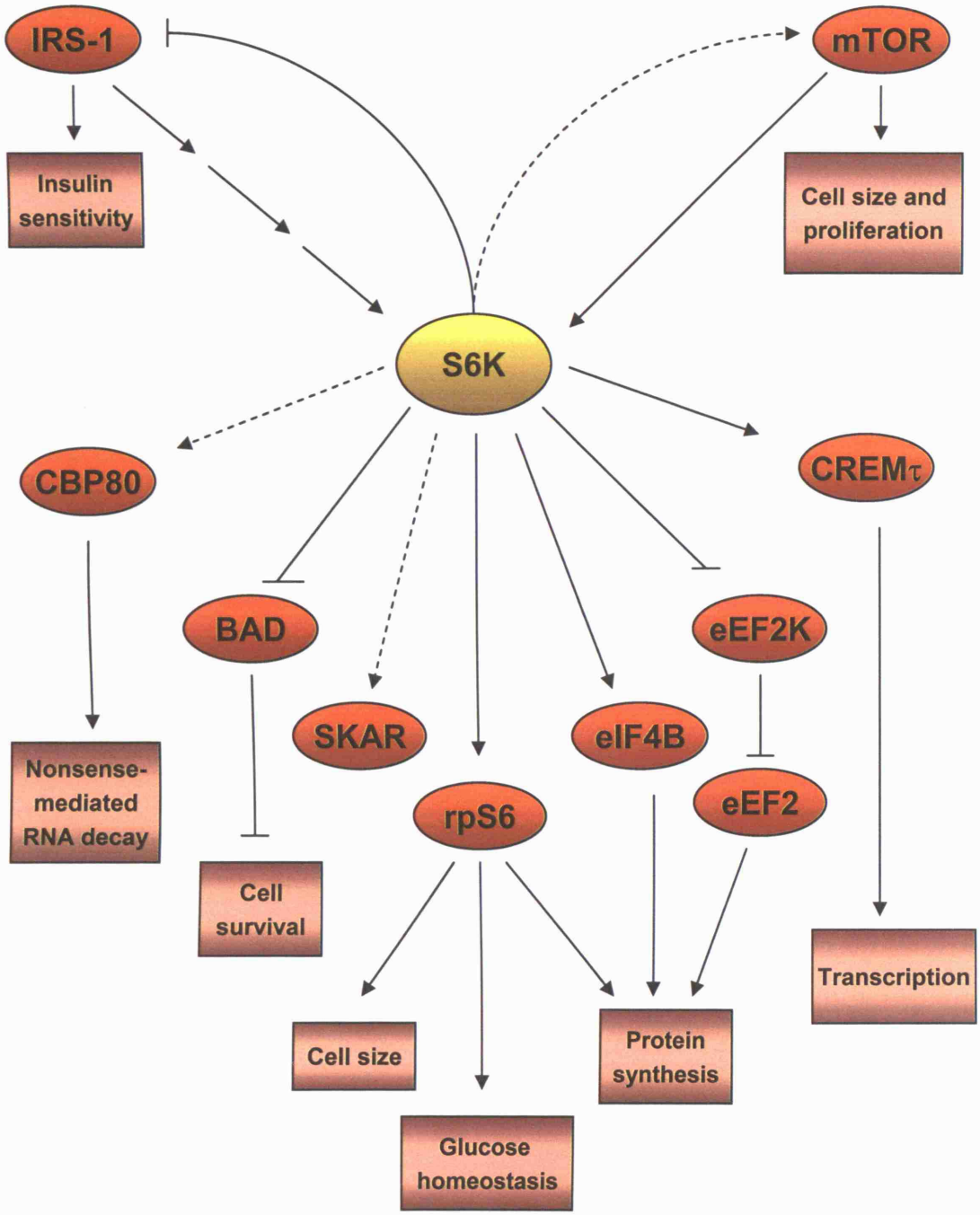


Fig. 1.8 Downstream targets and cellular effects of S6K.

Ribosomal protein S6 kinase has several effectors. Phosphorylation by S6K leads to activation of ribosomal protein S6 (rpS6), the transcription factor CREMt, and eukaryotic initiation factor 4B (elF4B), in addition to inhibition of IRS-1, the proapoptotic protein BAD, and eukaryotic elongation factor 2 kinase (eEF2K). S6K has no assigned effect on SKAR, and CMP80. S6K phosphorylates IRS1 and mTOR in a feedback regulatory manner, whereas its effect on mTOR is still unclear. The cellular processes regulated by each of the S6K effectors are boxed.

within their 5'UTR. This family of mRNA is referred to as 5'TOP mRNAs, and typically makes up 20-30% of the total cellular mRNA. 5'TOP mRNAs encode exclusively for components of the translational apparatus, including all ribosomal proteins, elongation factors, and poly(A)-binding proteins (PABP). Hence, increasing translation of 5'TOP mRNAs not only increase protein content directly, but also increase the translational capacity of cells (Terada *et al.*, 1994;Jefferies *et al.*, 1997).

The notion that S6K1 regulates the translation of 5'TOP mRNAs was initially proposed when interfering mutants of S6K1 were shown to impair mitogen-induced translation of 5'TOP mRNAs (Terada *et al.*, 1994;Jefferies *et al.*, 1997). A rapamycin-resistant S6K1 mutant confers rapamycin resistance to the translation of 5'TOP mRNAs (Schwab *et al.*, 1999). On this basis, S6 protein phosphorylation is generally a good readout for S6K activity as it correlates well with the translational activation of 5'TOP mRNAs. It was hypothesized that S6 phosphorylation is required to recruit 5'TOP mRNAs to ribosomes.

The requirement for S6K1 activity in translation of 5' TOP-containing mRNAs has been recently challenged. Whereas it is established that inactivation of S6K1 decreases synthesis of ribosomal proteins and translation factors (Terada *et al.*, 1994;Jefferies *et al.*, 1997), neither phosphorylation of ribosomal S6 protein nor activation of S6Ks is sufficient to relieve translational repression of 5'TOP mRNAs. Moreover, expression of dominant-negative S6K1 mutants failed to suppress translation in amino-acid-stimulated cells, and 5'TOP mRNAs were translationally regulated by amino acids in embryonic stem cells that lacked both alleles of S6K1 (Tang *et al.*, 2001). Meyuhas and colleagues provided further evidence that neither rpS6 phosphorylation nor S6K1 are essential for the translational regulation of 5' TOP mRNAs (Stolovich *et al.*, 2002). Moreover, complete inhibition of mammalian target of rapamycin (mTOR) and its effector S6K by rapamycin in various cell lines, resulted in only a mild repressive effect on the translation of 5' TOP mRNAs (Tang *et al.*, 2001). However, this function remained fully sensitive to PI3K inhibitors, wortmannin and LY290004. Based

on these data, it was proposed that translation of 5' TOP mRNAs is primarily regulated by growth and mitogenic cues through the PI3-kinase pathway, with a minor role, if any, for the mTOR pathway (Ruvinsky and Meyuhas, 2006). The knock-out model of both isoforms of S6K clearly indicates that S6K signalling is involved in the regulation of glucose homeostasis, cell size and growth, but is dispensable for translational control of mRNAs with a 5' TOP mRNAs (Pende *et al.*, 2004).

1.3.1.2 The role of S6K signalling in the initiation of translation

The eukaryotic translation initiation factor 4B (eIF4B) is another physiologically relevant target to S6K1 that could explain its effect on translation and cell growth. eIF4B is an RNA-binding protein that specifically stimulates the ATPase and RNA helicase activity of eIF4A, and is also required for efficient recruitment of ribosomes to mRNAs. eIF4B is phosphorylated in response to a variety of extracellular stimuli, such as serum, insulin, and phorbol esters which promote cell growth and proliferation (Duncan and Hershey, 1985). Phosphorylation of Ser422 in eIF4B was found to be specifically mediated by S6K1/2 *in vitro* (Raught *et al.*, 2004). *In vivo* results are consistent with Ser422 as a target of S6K1/2. Phosphorylation of this site is sensitive to wortmannin and LY290004, which block the signaling from the PI3K pathway; and rapamycin, a specific inhibitor of the mTOR pathway. Moreover, rapamycin-resistant S6Ks confer rapamycin resistance upon Ser422 phosphorylation *in vivo*. Consistent with these results, Ser422 phosphorylation is significantly decreased in S6K1/2 double knockout cells (Raught *et al.*, 2004).

It has been suggested that increased phosphorylation enhances both eIF4B activity, and the translation of mRNAs containing some degree of secondary structure. The use of a ribosome footprinting assay has also demonstrated that eIF4B is required for ribosome binding of mRNAs containing secondary structure (Dmitriev *et al.*, 2003). The recognition that S6K phosphorylates eIF4B

suggests a regulatory role for S6K in translation initiation, especially for mRNAs with complex structures at their 5'UTR.

1.3.1.3 The role of S6Ks in the elongation of translation

The polypeptide chain is extended during elongation, a phase of translation that could potentially exhaust the cells limited energy and aminoacyl tRNAs. Elongation requires eEF2, a 93kDa GTP-binding protein that mediates the translocation step of peptide-chain elongation, which is also understood to be a target for mTOR signalling (Proud, 2004). Phosphorylation of eEF2 at Thr56 by eEF2 kinase (eEF2K), a calcium/calmodulin dependent protein kinase, interferes with the binding of eEF2 to the ribosome and thus impairs the translocation step.

mTOR indirectly regulates eEF2 by modulating the activity of eEF2 kinase. eEF2 kinase is regulated by insulin via a rapamycin-sensitive pathway, implying the eEF2 kinase is downstream of mTOR. One mTOR-dependent input to eEF2 kinase is through S6K1 (Wang *et al.*, 2001c). *In vitro* S6 kinase assay showed that eEF2K is phosphorylated by S6K1, and the phosphorylation site was mapped to Ser366. In agreement with these results, mutant eEF2K proteins in which Ser366 was mutated to Ala, were not phosphorylated by S6K1. Moreover, eEF2K activity was clearly reduced following pre-treatment with S6K1. These data demonstrate that S6K1 phosphorylates eEF2K at Ser366, resulting in a significantly diminished level of activity. This inhibitory effect through S6K1 on eEF2K could release its phosphorylation and repression on eEF2, as loss of S6K1 greatly impaired the ability of IGF-1 to cause dephosphorylation of eEF2 (Wang *et al.*, 2001c).

Taken together, these data indicate that S6K phosphorylates a rapamycin-sensitive site, Ser366, on eEF2K, thus inhibiting its activity. So, as well as increasing the translational capacity and triggering translation initiation in cells, S6K also

mediates elongation machinery by enhancing eEF2 activity.

1.3.2 mRNA biogenesis and S6Ks

S6K signalling is also thought to regulate post-transcriptional processes important for mRNA biogenesis. For example, the RNA splicing and export factor CBP80, a subunit of nuclear cap binding complex, was reported to be a substrate of S6K1 (Wilson *et al.*, 2000). Richardson and coworkers have identified a novel S6K1 - but not S6K2 - substrate, named SKAR (Richardson *et al.*, 2004); SKAR is a nuclear protein with homology to the Aly/REF family of RNA binding proteins, which have been proposed to couple transcription with pre-mRNA splicing and mRNA export. The interaction between SKAR and S6K1 and the phosphorylation of SKAR by S6K1 suggests a potential role for S6K1 in post-transcriptional control of mRNAs.

1.3.3 Control of cell cycle progression, cell growth, and cell proliferation

Multiple lines of evidence indicate that S6Ks play an important role in progression through the G1 phase of the cell cycle. In general, cell size is very much uniform within the same tissue or organ of an equivalent species. Furthermore, cells need to grow to a certain size before they can advance through the cell cycle. Signalling pathways involved in the regulation of cell size and cell cycle progression have been studied extensively in the last decade. S6K has emerged in these studies as an important player, both in growth signalling and G1/S transition of the cell cycle.

The involvement of S6K in the regulation of the cell cycle was originally demonstrated in Thomas' laboratory. By microinjecting quiescent rat embryo

fibroblasts with three distinct polyclonal antibodies to S6K (which exhibit blocking activities), they observed the abrogation of serum-induced entry into S phase of the cell cycle. It was also found that the cell cycle block at the G1/S transition is preceded by an almost complete block of the activation of protein synthesis in response to serum stimulation (Lane *et al.*, 1993). Following this discovery, the importance of the nuclear isoform of S6K1 (p85-S6K1) in the control of serum-induced G1/S transition of the cell cycle was also demonstrated (Reinhard *et al.*, 1994).

S6Ks also regulate the rate of cell cycle progression. When quiescent U2OS cells are stimulated with serum to enter G1 phase from G0, overexpression of S6K1 accelerates S phase entry, while reduced expression of S6K1 with RNAi inhibits the rate of S phase entry (Fingar *et al.*, 2004). Moreover, overexpression of rapamycin-resistant forms of S6K1 partially rescues the rapamycin-induced delay in G1 progression to S phase, indicating S6K1 to be one of the mediators of mTOR-dependent cell division (Fingar *et al.*, 2004). These data are consistent with the ability of rapamycin-resistant mutants of S6K1 to partially restore rapamycin-suppressed E2F-dependent transcription and to partially rescue rapamycin-inhibited proliferation of vascular smooth muscle cells (Brennan *et al.*, 1999;Vinals *et al.*, 1999). Furthermore, embryonic stem cells lacking S6K1 proliferate in culture at a reduced rate (Kawasome *et al.*, 1998). These data suggests a role for S6K in cell cycle and cell proliferation regulation.

Knockout studies in *Drosophila* showed that S6K-deficient fruit flies are smaller than wild-type flies (Montagne *et al.*, 1999). Consistent with this study, S6K1-knockout mice are also around 20% smaller than wild-type littermates (Shima *et al.*, 1998). Unlike PI3K pathway that controls both cell size and number, the reduced animal size in S6K knockouts is due to a decrease in cell size rather than a reduction in cell numbers (Potter and Xu, 2001;Pende, 2006). Moreover, overexpression of S6K1 rescued the TOR-knockout phenotype, suggesting that the loss of signalling to S6K accounts for the cell size phenotype of the TOR-deficient fruit flies (Zhang *et al.*, 2000). These reports clearly show

that the S6 kinase plays a key role in growth control.

1.3.4 Cell survival and S6K signalling

An anti-apoptotic function of S6K1 has been suggested based on its ability to phosphorylate and inactivate pro-apoptotic protein BAD (Harada *et al.*, 2001). BAD forms a heterodimer with Bcl-2 and Bcl-X_L at the mitochondrial membrane to promote intrinsic apoptosis, mediated by mitochondria through the release of apoptosis promoting factors (Green and Reed, 1998). When phosphorylated on Ser112 or Ser136, BAD no longer interacts with Bcl-2 and Bcl-X_L, allowing them to inhibit cell death.

It was previously shown that the pro-apoptotic function of BAD was regulated by the PI3K-PKB/Akt pathway. Interleukin 3-induced PKB/Akt activation leads to phosphorylation of BAD on Ser136 and abrogates its apoptotic function by causing 14-3-3-mediated sequestration in the cytosol (del Peso *et al.*, 1997). However, Harada and coworkers have shown that BAD phosphorylation is not significantly reduced in cells from which PKB/Akt has been immunodepleted (Harada *et al.*, 1999). They went on to show that S6K1 was responsible for IGF-1-induced BAD Ser136 kinase activity purified from outer mitochondrial membranes. The mTOR/S6K inhibitor, rapamycin, inhibits IGF-1-induced BAD phosphorylation and cell survival. Moreover, this model was supported by experiments using S6K1$^{-/-}$ ES cells, which displayed no IGF1-induced BAD phosphorylation (Harada *et al.*, 2001). Thus, while PKB clearly has a role in cell survival signalling through targeting multiple substrates, under some conditions it appears that S6K1 activity makes an important contribution to this process.

Additional proof of the involvement of S6Ks in the regulation of cell survival has been recently provided by Pardo and colleagues (Pardo *et al.*, 2006). In this study, the survival effect of FGF2 on small cell lung cancer cells was found to be

mediated by S6K2, complexed with PKC epsilon and B-Raf.

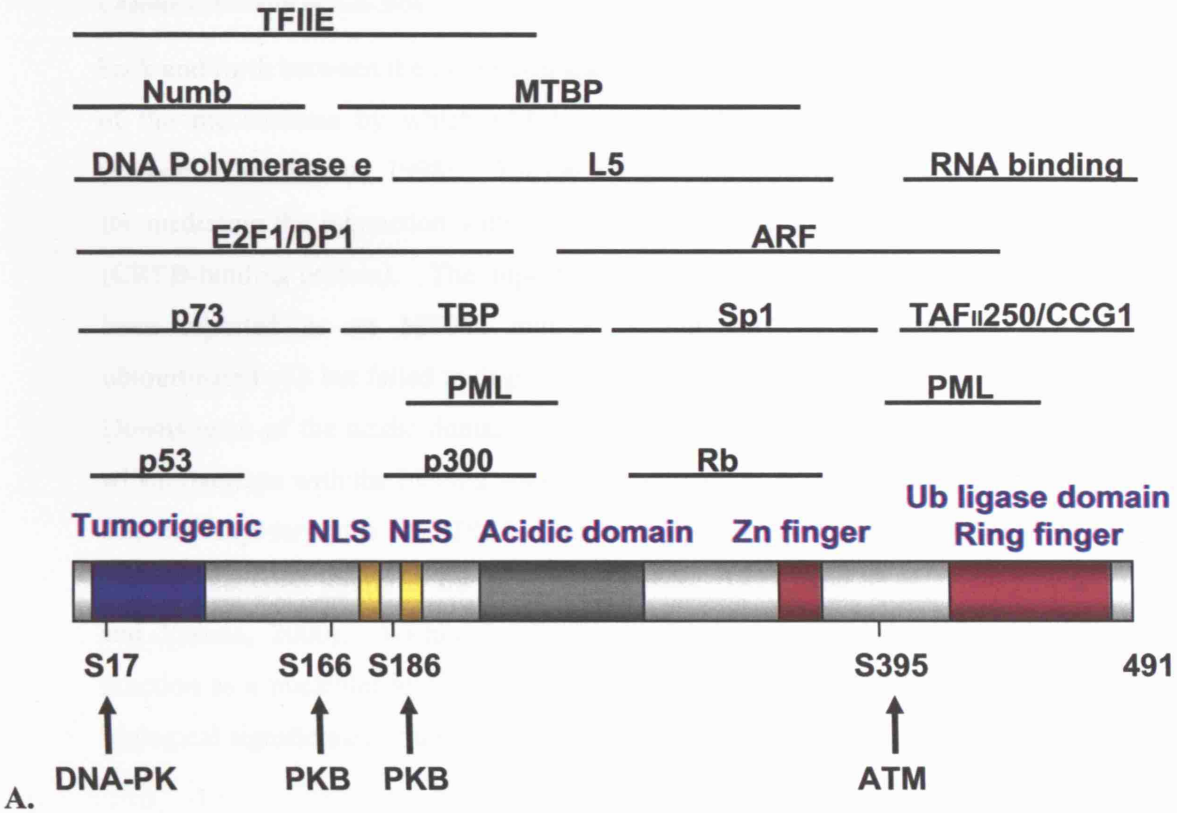
1.4 Signalling networks involving MDM2

The murine double minute 2 (*mdm2*) gene was originally identified as one of three genes (*mdm1, 2*, and *3*) overexpressing more than 50-fold, by amplification in a spontaneously transformed mouse BALB/c cell line (3T3-DM). The *mdm* genes are located on small, acentromeric extrachromosomal nuclear bodies, called double minutes, which are only retained in cells if they provide benefits for growth. The gene product of the *mdm2* gene was later shown to be responsible for the transformation of cells when overexpressed (Cahilly-Snyder *et al.*, 1987;Fakharzadeh *et al.*, 1991).

Soon after the identification of the *mdm2* gene, the reason for its transformation potential was discovered. MDM2 protein was shown to bind to the tumor suppressor p53 and inhibit p53-mediated transactivation, a stimulation of transcription by a transcription factor binding to DNA (Momand *et al.*, 1992;Oren *et al.*, 2002;Agrawal *et al.*, 2006;Shu *et al.*, 2007). In addition, *mdm2* gene amplification was observed in over one third of human sarcomas that retained wild-type p53 (Oliner *et al.*, 1992). Although rare, some tumors contain both high levels of MDM2 protein and mutations in the *p53* gene.

1.4.1 Domain organization of MDM2

MDM2 is a nuclear phosphoprotein containing several conserved functional regions. A schematic representation of the MDM2 domains is presented in Figure 1.9a. The amino-terminus contains the p53-interacting domain, which is located between amino acid residues 23-108 (Chen *et al.*, 1993;Kussie *et al.*, 1996). This domain binds the amino terminal transactivation domain of p53, thus interfering with p53 interaction with the transcriptional machinery. Further downstream from the p53-interaction domain, lies a nuclear localization sequence (NLS) and a nuclear export sequence (NES). These sequences shuttle MDM2



A.

B.

Fig. 1.9 Domain organization, regulatory sites of phosphorylation and regions mediating protein-protein interactions in MDM2.

A. The MDM2 protein is a nuclear phosphoprotein that contains several conserved functional regions. The N-termimus contains the p53-interacting domain, located between residues 23-108. There are nuclear localization sequence (NLS), nuclear export sequence (NES), and a zinc finger domain in the central region. The C-terminus of MDM2 contains a RING finger domain which possesses E3 ubiquitin ligase activity mediating p53 degradation. In addition to p53, many other MDM2-interacting proteins have been recently identified. These include p73, one of the p53 family protein, DNA polymerase e, the transcription factor E2F1/DP1, Numb, and TFIIE binding to the N-terminus of MDM2; TAFII250/CCG1 and PML binding to the C-terminus. Within the central domain, there are binding sites for TBP, MTBP, PML, Sp1, the ribosomal protein L5, the transcriptional coactivator p300, the Rb tumor suppressor protein, and ARF, one of two products encoded by overlapping reading frames within the INK4A gene, which binds to MDM2 and blocks its ability to mediate ubiquitination of p53. **B.** Two consensus sequences, located near the nuclear localization and nuclear export motifs, for Akt-mediated phosphorylation are highlighted.

back and forth between the cytoplasm and nucleus, providing yet another example of the mechanisms by which p53 transcriptional activity is tightly regulated (Freedman and Levine, 1998). The central acidic domain of MDM2 is necessary for mediating the interaction with the ribosomal protein L5, TBP, and p300/CBP (CREB-binding protein). The importance of this domain in p53 degradation has been reported as an MDM2 mutant lacking part of this region, which ubiquitinated p53 but failed to degrade it (Argentini *et al.*, 2001;Zhu *et al.*, 2001). Downstream of the acidic domain is a zinc finger domain of unknown function, which overlaps with the binding sites for tumor suppressor proteins RB and ARF. The carboxyl-terminus of MDM2 contains a RING finger domain, required for MDM2-mediated ubiquitin transfer (Honda *et al.*, 1997;Fang *et al.*, 2000;Honda and Yasuda, 2000). Within the RING finger domain, amino acids 464-471 function as a nucleolar localization signal (Lohrum *et al.*, 2000). However, the biological significance of this signal is still unclear.

1.4.2 The negative feedback loop between p53 and MDM2

Since 1979, and the discovery of p53 as an SV40 large T antigen-binding protein, it has been the subject of intense study. It has known since 1989, that mutations in p53 are associated with human cancers (Baker *et al.*, 1989;Takahashi *et al.*, 1989;Nigro *et al.*, 1989). Around 50% of human cancers carry mutated p53, and human tumor cells which retain wild-type p53 often have defects in activating or responding to p53. p53 is a sequence-specific transcription factor, as well as a major guardian of the genome (Alarcon-Vargas and Ronai, 2002;Oren *et al.*, 2002;Agrawal *et al.*, 2006). In response to stresses such as DNA damage and abnormal proliferation, p53 is accumulated and activated within cells, leading to cell cycle arrest or apoptosis by triggering the expression of molecules which regulate these processes. p53 facilitates the repair of damaged DNA, and eliminates irreversibly damaged or abnormally growing cells to prevent potential transformation. Under certain conditions, blocking the cell cycle protects cells from programmed death, while a different set of circumstances mean that growth

arrest and apoptosis occurs.

MDM2 was identified as one of the transcriptional targets of p53. p53 binds the *mdm2* P2 promoter and transcriptionally up-regulates *mdm2* expression. Figure 1.10 shows a current model of the autoregulatory feedback mechanism between MDM2 and p53. As p53 is a potent cell cycle inhibitor and an inducer of apoptosis, its level and activity are expected to be under tight control. MDM2, the expression of which can be enhanced by p53 at the transcription level, is responsible for low p53 levels in unstressed cells (Agrawal *et al.*, 2006;Shu *et al.*, 2007). Studies from numerous laboratories indicate that MDM2 inhibits p53 in two ways: by inducing p53 degradation through the ubiquitin-proteasomal pathway, or by interfering with p53 transactivation function through direct binding (Oren *et al.*, 2002;Agrawal *et al.*, 2006;Shu *et al.*, 2007). MDM2 possesses the activity of an E3 ubiquitin ligase in its carboxyl-terminal RING finger domain, which mediates autoubiquitination as well as the ubiquitination of other substrates, including p53 (Honda *et al.*, 1997;Fang *et al.*, 2000;Honda and Yasuda, 2000;Agrawal *et al.*, 2006). MDM2 binds to the amino terminus of p53 via its amino-terminal p53-interaction domain, and covalently attaches ubiquitin, a 76-amino acid protein, to lysine residues in the carboxyl-terminus of p53 (Chen *et al.*, 1993;Kussie *et al.*, 1996;Rodriguez *et al.*, 2000;Agrawal *et al.*, 2006). This process occurrs mainly in the nucleus, where both proteins are localized (Boyd *et al.*, 2000;Yu *et al.*, 2000;Geyer *et al.*, 2000). Although some reports have claimed the existence of nuclear degradation of p53 (Joseph *et al.*, 2003), it is generally believed that translocation from the nucleus to the cytoplasm is required for the ubiquitinated MDM2/p53 complex to be degraded by the 26S proteasomes (Freedman and Levine, 1998;Roth *et al.*, 1998;Tao and Levine, 1999a;Tao and Levine, 1999b;Boyd *et al.*, 2000;Geyer *et al.*, 2000;Agrawal *et al.*, 2006;Shu *et al.*, 2007). On the other hand, the interaction between p53 and MDM2 could disrupt binding between p53 and its target genes, since both interactions compete with an overlapped region at the amino-terminus of p53, thus directly concealing it from basic transcriptional machinery and coactivators (Momand *et al.*, 1992;Agrawal *et al.*, 2006;Shu *et al.*, 2007).

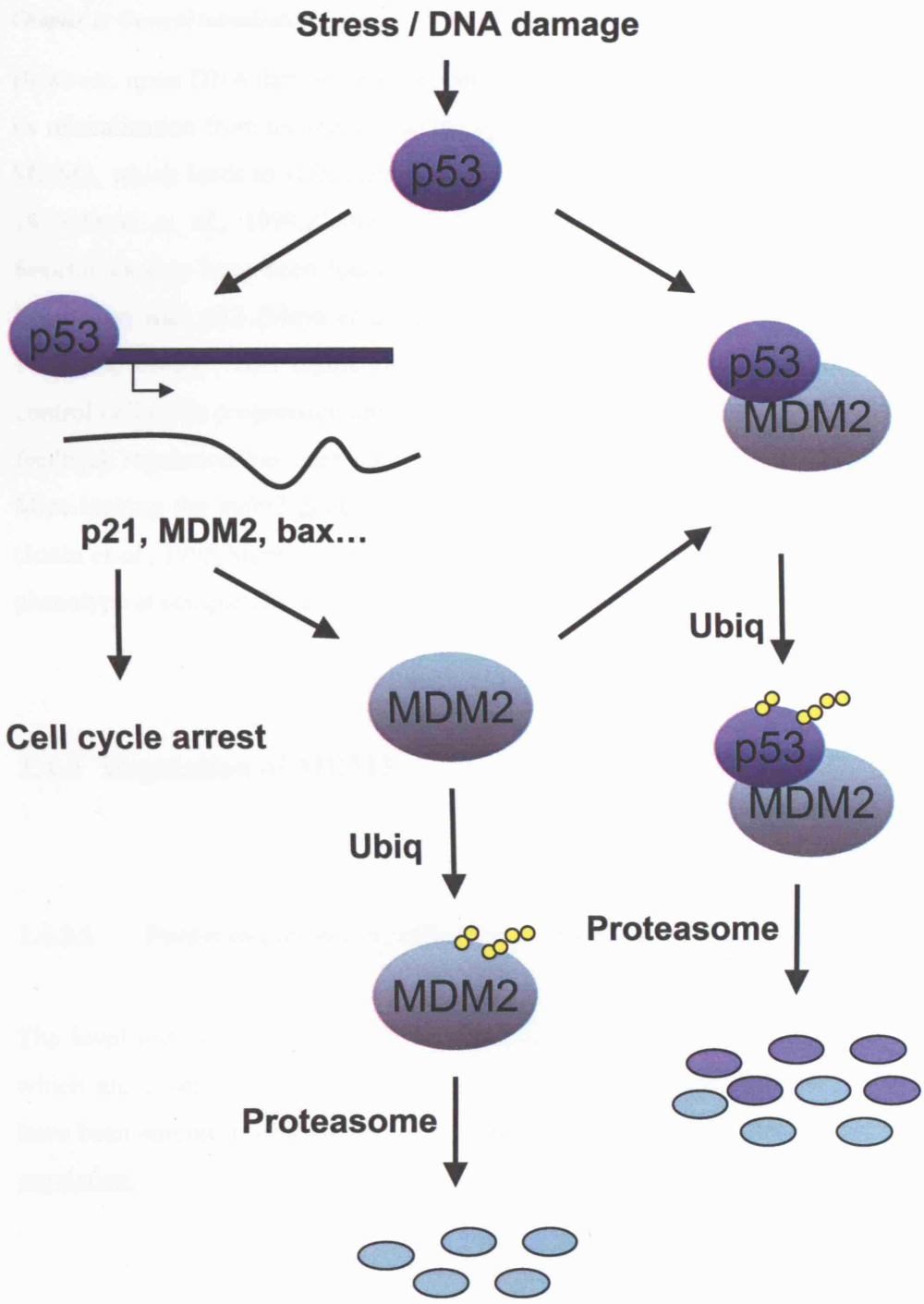


Fig. 1.10 MDM2/p53 autoregulatory feedback loop mechanism.

A schematic model presenting the functional interaction between MDM2 and p53. MDM2 is one of the transcriptional targets of p53. According to this model, p53 induces the expression of the MDM2 protein, which itself is very unstable due to its auto-ubiquitination activity. MDM2 interacts with p53, which interferes with the interaction of p53 with the transcriptional machinery, as well as mediates p53 ubiquitination. The ubiquitinated MDM2 and p53 proteins are degraded by proteasomes, inhibiting pro-apoptosis signalling.

However, upon DNA damage, p53 is post-translationally modified, which inhibits its relocalization from the nucleus to the cytoplasm, and also its interactions with MDM2, which leads to stabilization of p53 (Siliciano *et al.*, 1997;Chehab *et al.*, 1999;Araki *et al.*, 1999;Zhang and Xiong, 2001;Moll and Petrenko, 2003). Several kinases have been found to phosphorylate MDM2 and to modulate its interaction with p53 (Maya *et al.*, 2001;Meek and Knippschild, 2003;Moll and Petrenko, 2003). This regulation is an important role for p53, enabling it to control cell cycle progression and apoptosis. The importance of the MDM2/p53 feedback regulation has been convincingly demonstrated in *in vivo* experiments. Mice lacking the *mdm2* gene are embryonic lethal and die before implantation (Jones *et al.*, 1995;Montes de Oca *et al.*, 1995). This is due to active p53, as the phenotype is completely rescued by concomitant deletion of *p53*.

1.4.3 Regulation of MDM2

1.4.3.1 Post-translational modifications regulate MDM2 functions

The level and activity of MDM2 is regulated by a variety of signals, most of which are involved in multisite phosphorylation of MDM2. Recently, reports have been emerging which indicate the importance of phosphorylation in MDM2 regulation.

1.4.3.1.1 DNA-PK signals to MDM2

The DNA-activated protein kinase is a member of the PIKK which includes the ATM (ataxia telangiectasia-mutated), ATR (ATM and Rad3-related) and mTOR protein kinases. MDM2 is phosphorylated *in vitro* by both DNA-PK and ATM (Mayo *et al.*, 1997;Khosravi *et al.*, 1999;de Toledo *et al.*, 2000;Meek and

Knippschild, 2003), but phosphorylation by ATR has not yet been reported.

Of eight potential DNA-PK targets, MDM2 was shown to be phosphorylated by this enzyme at Ser17 both *in vitro* and *in vivo* (Mayo *et al.*, 1997). The phosphorylation itself was reported to have a significant impact on the ability of MDM2 to regulate the p53 response. For example, ELISA analysis has shown that phosphorylation of MDM2 at Ser17 can block the MDM2-p53 interaction *in vitro*, whereas the ability of a S17A mutant of MDM2 to associate with p53 is unaltered following phosphorylation by DNA-PK (Mayo *et al.*, 1997). Consistent with this observation, an S17A mutant was shown to be significantly more effective in inhibiting p53-dependent transactivation in cultured cells than wild-type MDM2. On the other hand, the Ser17 site is located in close proximity to the amino-terminal p53-interacting domain of MDM2, which may suggest that phosphorylation of this site structurally affects the affinity of MDM2-p53 association.

1.4.3.1.2 ATM regulates MDM2 functions by phosphorylation of Ser395

ATM mediates the phosphorylation of MDM2 at Ser395, located in one of the two epitopes recognized by monoclonal antibody 2A10 (Coutts and La Thangue, 2006). Consistent with the identification of this site, a decrease of 2A10 affinity to MDM2 was observed in an ATM-dependent manner.

Direct phosphorylation of MDM2 at Ser395 by ATM inhibits its ability to mediate p53 turnover (Khosravi *et al.*, 1999;de Toledo *et al.*, 2000;Maya *et al.*, 2001), as microinjection of 2A10 is sufficient to induce p53. An S395D mutant (where the substitution of Asp for Ser was carried out to mimic the negative charge provided by a phosphate) was able to weakly degrade p53 in comparison to wild-type MDM2 or an S395A mutant, and was unable to promote nuclear export of p53.

ATM is also suspected to indirectly regulate the phosphorylation of other residues in MDM2, induced by other kinases. The finding that the target of c-Abl, Tyr394, is immediately adjacent to the ATM target, Ser395, raise the interesting possibility that c-Abl- and ATM-dependent phosphorylation may work in a closely regulatory manner (Sionov *et al.*, 2001;Goldberg *et al.*, 2002;Meek and Knippschild, 2003).

1.4.3.1.3 PI3K pathways regulates MDM2 via PKB/Akt phosphorylation at Ser166 and Ser 186

Several groups have published details of the physical and functional interaction between MDM2 and PKB/Akt (Zhou *et al.*, 2001;Mayo and Donner, 2001;Ashcroft *et al.*, 2002;Ogawara *et al.*, 2002;Gottlieb *et al.*, 2002;Feng *et al.*, 2004;Milne *et al.*, 2004). Following mitogen-induced activation, PKB/Akt appears to associate with and phosphorylate MDM2 at Ser166 and Ser186 (Zhou *et al.*, 2001;Mayo and Donner, 2001). Phosphorylation by PKB/Akt has been shown to affect MDM2 functions such as subcellular localization, MDM2-p53 association, MDM2-p300 association, E3 ligase activity, and MDM2 level in cells. Figure 1.11 outlines the effect of MDM2 phosphorylation via the PI3K-PKB/Akt pathway on p53 signalling.

The MDM2-PKB/Akt association was found to be concurrent with the phosphorylation of PKB/Akt at Thr473, which is generally a readout of PKB/Akt activity, suggesting a positive regulatory role of PKB/Akt activity on this protein-protein interaction (Ashcroft *et al.*, 2002). Phosphorylation of MDM2 increases MDM2 levels, which is possibly mediated through the inhibition of MDM2 self-ubiquitination (Feng *et al.*, 2004). Immunofluorescence analysis indicated that phosphorylation of Ser166 and Ser186 promotes MDM2 relocalization from cytoplasm to nucleus, where it binds to p53, therefore enhancing p53 ubiquitination and degradation (Zhou *et al.*, 2001;Ashcroft *et al.*, 2002). The discovery of PKB/Akt's role in MDM2 regulation reveals a

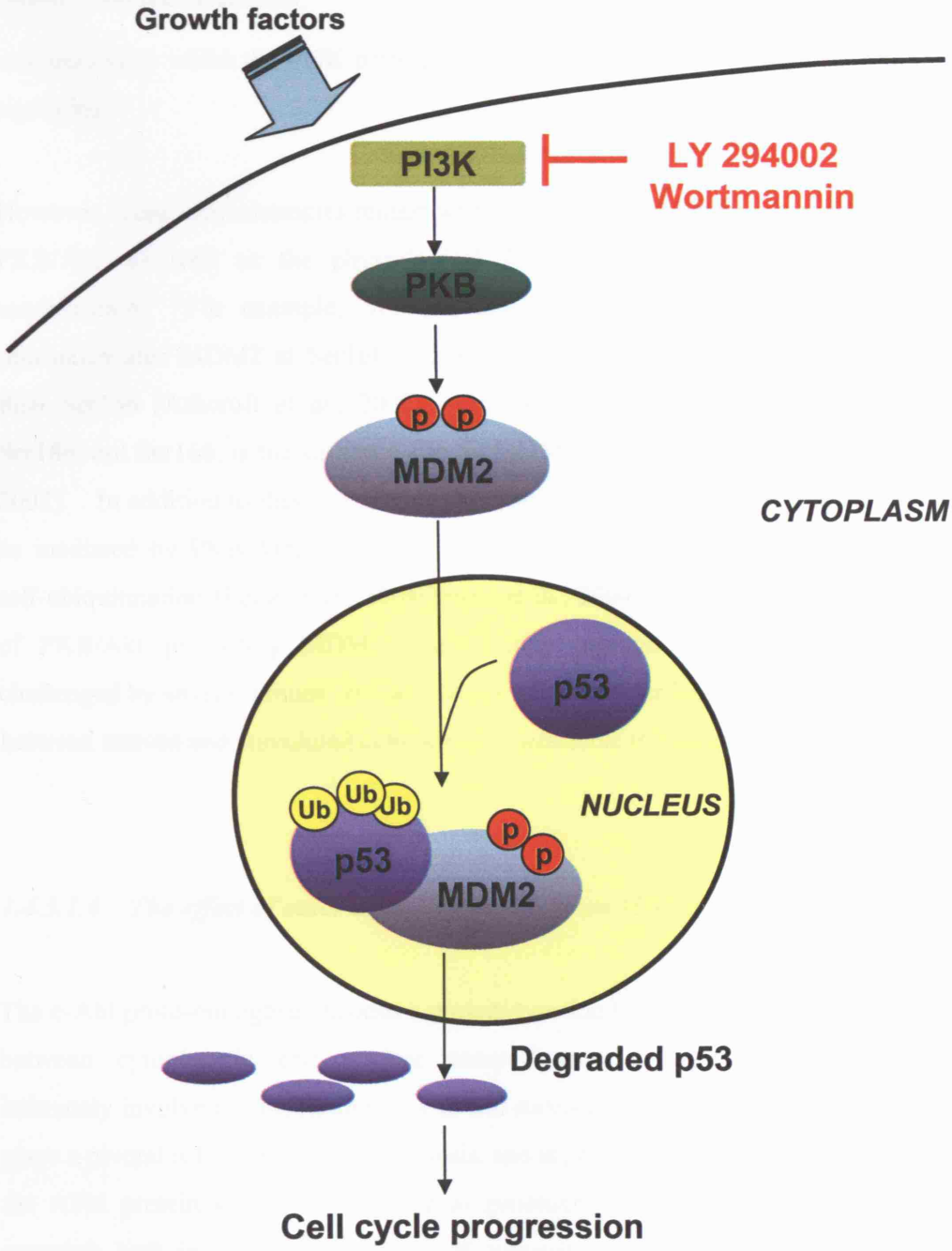


Fig. 1.11 Regulation of MDM2 by PI3K/PKB pathway.

Survival factors acting through the PI3K pathway lead to the phosphorylation and activation of PKB/Akt. According to the current model, PKB associates with MDM2 in the cytoplasm and phosphorylates MDM2 at Ser166 and Ser186. Both phosphorylation sites have been implicated in regulating subcellular localization of MDM2 and stimulating its ability to ubiquitinate p53. Ubiquitination of p53 enhances its degradation and promotes cell cycle progression. Details of this mechanism are given in the text.

mechanism by which the PI3K pathway may be able to mediate its anti-apoptotic signalling.

However, some inconsistencies remain with regards to MDM2 phosphorylation by PKB/Akt, as well as the physiological functions of this post-translational modification. For example, Vousden and colleagues stated that PKB/Akt phosphorylates MDM2 at Ser166 and at additional phosphorylation sites other than Ser186 (Ashcroft *et al.*, 2002). Additionally, another study claims that Ser186, not Ser166, is the substrate site on MDM2 for PKB/Akt (Ogawara *et al.*, 2002). In addition to these sites, phosphorylation of Ser188 was also claimed to be mediated by PKB/Akt, leading to the stabilization of MDM2 via decreased self-ubiquitination (Feng *et al.*, 2004;Milne *et al.*, 2004). Furthermore, the idea of PKB/Akt promoting MDM2 relocalization through phosphorylation was challenged by several groups, as the changes in subcellular localization of MDM2 between starved and stimulated cells was not detectable (Ogawara *et al.*, 2002).

1.4.3.1.4 The effect of other signalling pathways on MDM2

The c-Abl proto-oncogene encodes a protein-tyrosine kinase that is able to shuttle between cytoplasmic and nuclear compartments. Cytoplasmic c-Abl is intimately involved in mediating growth and survival signals, while nuclear c-Abl plays a pivotal role in mediating apoptosis, and is phosphorylated and activated by the ATM protein kinase in response to genotoxic agents. MDM2 and c-Abl associate both *in vitro* and in nuclei of cultured cells, which leads to MDM2 phosphorylation at multiple sites (Sionov *et al.*, 2001). Tyr394 was proposed and subsequently identified as a key site of c-Abl-dependent phosphorylation in this study. The Y394F mutant was shown to increase MDM2-mediated p53 degradation, to stimulate MDM2-mediated down-regulation of p53-dependent transactivation and apoptosis. These findings are in agreement with the model in which DNA damage-dependent phosphorylation of MDM2 contributes to apoptosis by blocking the ability of MDM2 to suppress p53 functions.

Studies on cyclinA/CDK2, also reveal the link with MDM2 phosphorylation in a cycle-dependent manner (Zhang and Prives, 2001). It seems that cyclinA/CDK2 phosphorylation of murine MDM2 at Thr216 shifts the MDM2-p53 interaction to MDM2-ARF interaction, which in turn stabilizes p53. However, the phosphorylation might be species-specific, as human MDM2 lacks the cyclin-CDK substrate recognition motif (CRM). CK2 was also reported to phosphorylate murine MDM2 at Ser267 (equivalent to Ser269 in human MDM2) and Ser258; but the biological significance of these phosphorylation events remains unclear (Hjerrild *et al.*, 2001).

A cluster of phosphorylation sites observed in the acidic domain has recently been shown to contain phosphorylated serine 240, 242, 246, 253, 256, 260, and 262 (Hay and Meek, 2000;Blattner *et al.*, 2002). These residues are normally phosphorylated in the cell under non-stressed conditions (Hay and Meek, 2000), and the modification of these residues may contribute to the promotion of MDM2-mediated p53 turnover or the alteration of MDM2's affinity to its binding partners. On the other hand, Blattner *et al.* have shown that key phospho-serine residues in this domain, including serine 240, 242, 260, and 262, became rapidly dephosphorylated in response to ionizing radiation in a manner that clearly precedes p53 (Blattner *et al.*, 2002). This result suggests that by removing the phospho group on some or all of these residues, stress signals could attenuate MDM2-mediated p53 degradation and thereby contribute to p53 accumulation.

1.4.3.1.5 Cyclin G1 mediates dephosphorylation of MDM2 at T216 and S166

In response to various stress conditions, stress kinases such as ATM are activated, resulting in the phosphorylation and activation of p53. A number of studies have shown that some of these stress kinases also phosphorylate MDM2, and that phosphorylated MDM2 demonstrates a reduced ability to interact with and inhibit p53 (Michael and Oren, 2002). Therefore, removal of an inhibitory phosphate from MDM2 is necessary for its activation.

Cyclin G1 was one of the earliest p53 target genes to be identified, but its precise function in the p53 pathway has remained elusive (Okamoto and Beach, 1994). Research in this has shown that cyclin G1 displays growth-promoting functions, and is expressed at a high level in regenerating hapatocytes and motoneurons, and in rapidly growing cancer cells (Skotzko *et al.*, 1995;Morita *et al.*, 1996). These roles may appear to be inconsistent with an involvement in mediating the p53 response. In addition, cyclin G1 can also increase the sensitivity of cancer cells to TNFα-induced apoptosis (Okamoto and Prives, 1999).

Recently, cyclin G1 protein was reported as a regulator for MDM2 by mediating its dephosphorylation (Okamoto *et al.*, 2002). Cyclin G1 is known to interact with the regulatory B' subunits of PP2A phosphatase (Okamoto *et al.*, 1996), an abundant serine/threonine phosphatase which performs a variety of biological functions in cells. The cyclinG1-PP2A interaction remained unclear until Prives and colleagues found that cyclin G1 recruits PP2A to dephosphorylate MDM2 (Okamoto *et al.*, 2002). Their data has provided convincing evidence that cyclin G interacts with the enzymatically active PP2A phosphatase. In addition, cyclin G directly interacts with MDM2 and can stimulate the ability of PP2A to dephosphorylate MDM2, thus serving as a recruitment factor. Specifically, they provide strong evidence that the PP2A holoenzyme is capable of dephosphorylating two sites in MDM2, Thr216 and Ser166, both *in vitro* and *in vivo*.

This dephosphorylation may lead to enhanced degradation of p53 as mouse embryo fibroblasts (MEF) lacking cyclin G were shown to contain both higher p53 levels and hyperphosphorylated MDM2 at T216 when compared to wild-type cells. Expression of cyclin G in the cyclin $G^{-/-}$ MEF cells restores the activity of MDM2 in the degradation of p53. These data suggest that cyclin G serves as a negative regulator of p53 by activating MDM2 through dephosphorylation, which also provides a mechanistic explanation for the observation that cyclin G expression is associated with growth promotion rather than arrest (Skotzko *et al.*, 1995;Okamoto *et al.*, 2002).

1.4.3.2 Regulation of MDM2 functions by protein-protein interactions

Interacting with other proteins provides another mechanism by which MDM2 activity can be modulated. Several proteins have been identified as binding partners of MDM2, which are responsible for modulating its functions. Here I will mainly focus on ARF, a small basic protein encoded by the INK4a locus that also encodes the cyclin-dependent kinase inhibitor p16INK4a (Okamoto *et al.*, 2002;Lowe and Sherr, 2003).

1.4.3.2.1 ARF inhibits MDM2 function through direct interaction

Shortly after its identification, both human and mouse ARF (p14ARF and p19ARF, respectively) were found to interact with MDM2 through its central region. This interaction has been shown to block MDM2-mediated p53 degradation (Zhang *et al.*, 1998;Kamijo *et al.*, 1998;Chin *et al.*, 1998;Agrawal *et al.*, 2006;Gallagher *et al.*, 2006). This was attributed to the direct inhibition of MDM2 E3 activity by ARF (Honda and Yasuda, 1999). Recently, it was shown that the expression of ARF also promotes MDM2-mediated ubiquitination of MDMX, which is important for the stability of MDM2. It is conceivable that stress-induced ARF may contribute to the inhibition of MDM2 E3 activity toward p53 (Pan and Chen, 2003;Agrawal *et al.*, 2006). Experimentally, overexpressed ARF resides in the nucleolus, and it was found that ARF could induce the nucleolar localization of MDM2. Due to the separation of MDM2 from p53, or possibly by preventing nuclear export of the MDM2-p53 complex, nucleolar localization of MDM2 by ARF could result in the inhibition of p53 degradation (Zhang and Xiong, 1999;Weber *et al.*, 1999;Tao and Levine, 1999b).

However, the essential role of nucleolar retention of MDM2 is challenged by the findings that some mutated ARFs do not induce growth arrest despite their ability

to retain MDM2 in the nucleolus (Korgaonkar *et al.*, 2002). In addition, induction of ARF leads to the stabilization of p53 and cell cycle arrest without the relocation of MDM2 to the nucleolus in certain cells (Llanos *et al.*, 2001;Lin and Lowe, 2001). Moreover, forms of ARF that do not accumulate in the nucleolus retain the capacity to stabilize MDM2 and p53 (Llanos *et al.*, 2001). Therefore, relocation to the nucleolus is not essential for the inhibition of MDM2 by ARF in all cells. ARF-mediated nucleolar localization of MDM2 for stabilizing p53 may be required only under certain circumstances.

1.4.3.2.2 Other binding partners which regulate MDM2 signalling

Like ARF, the ribosomal protein L11 binds MDM2 and can sequester it in the nucleolus, resulting in p53 stabilization (Lohrum *et al.*, 2003). In transfection experiments, additional L11 inhibits the degradation of p53 by MDM2. In U2OS cells, the addition of L11 causes an increase in G1 arrest. Both the levels of L11, and its localization within a cell, affect p53 activity through interaction with MDM2.

Similarly, hypoxia-inducible factor 1α (HIF-1α) also interacts with MDM2 and enhances p53 function (Chen *et al.*, 2003). It was shown that this interaction prevents nuclear export of p53, but provides another example of a protein that may physically prevent MDM2 from binding to p53.

p300 and CBP are acetyl transferases which have been shown to interact with MDM2, cooperating in the degradation of p53 (Grossman *et al.*, 1998;Kawai *et al.*, 2001). Until recently, MDM2 was believed to polyubiquitinate p53 for protein degradation. However, recent data indicate that MDM2 mediates monomeric p53 ubiquitination on multiple lysine residues instead of polymeric ubiquitination (Lai *et al.*, 2001). Generally, monoubiquitination is believed to mediate receptor endocytosis, virus budding, transcription, DNA repair, and caspase recruitment in

apoptosis, while polyubiquitination leads to protein degradation through the proteosomal pathway (Lee and Peter, 2003;Yang and Yu, 2003). A chain of at least four ubiquitin molecules is believed to be necessary for efficient proteosomal degradation (Thrower *et al.*, 2000). *In vitro*, p300/CBP is required for polyubiquitination of p53, interacting and cooperating with MDM2 in the degradation of p53 (Grossman *et al.*, 2003). Phosphorylation of MDM2 by PKB/Akt, enhances its nuclear localization and interaction with p300/CBP, as well as inhibiting interaction with ARF, and increasing p53 degradation (Zhou *et al.*, 2001;Ashcroft *et al.*, 2002;Ogawara *et al.*, 2002). MDM2 mutants which lack part of the central acidic domain required for p300/CBP binding, fail to degrade p53 but accumulate monoubiquitinated p53 (Zhu *et al.*, 2001). This is an unusual role for p300/CBP because it was originally identified as a transcriptional coactivator with acetyltransferase activity (Shikama *et al.*, 1999), however, this acetyl transferase activity is not required for stabilization of MDM2 (Zeng *et al.*, 2003).

1.4.4 MDM2 associates with several cell cycle-regulating molecules

Several other cellular proteins have been identified in various experimental systems as binding partners for MDM2 (Figure 1.12). Using yeast, two hybrid screens or immunoprecipitation experiments, investigators identified many additional MDM2 interacting partners that are regulated by MDM2. In most cases, the interaction has not been observed in multiple systems, and these studies await further experiments to verify the specificity of binding at the physiological level. Among the identified binding partners, molecules that associate with cell cycle regulation have been more thoroughly characterized and are under intense investigation.

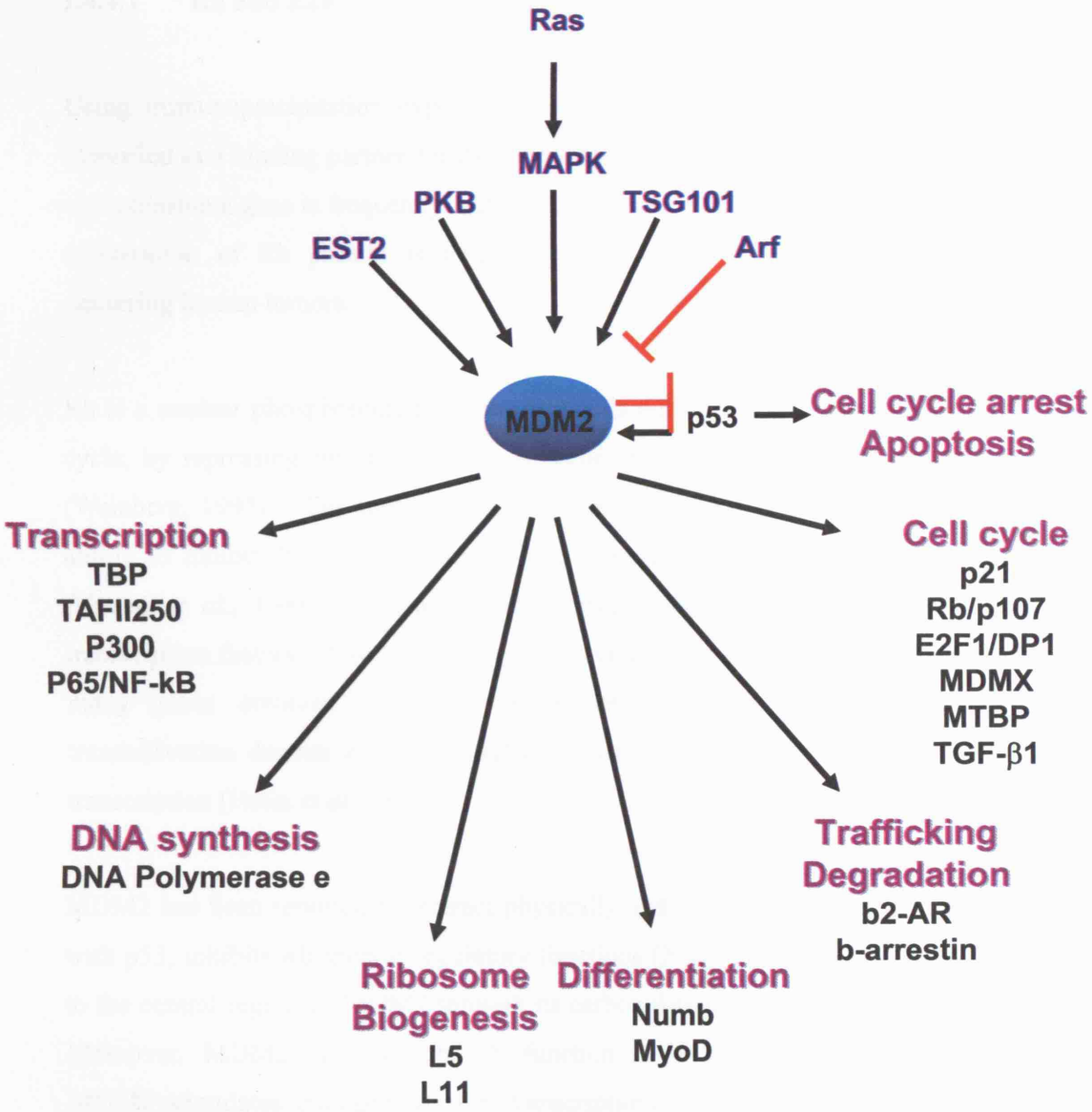


Fig. 1.12 Downstream signalling and regulation of cellular functions by MDM2.

MDM2 has been implicated in diverse cellular functions: a) regulation of transcription; b) DNA synthesis; c) ribosomal biogenesis; d) cell cycle; e) apoptosis; f) differentiation and malignant transformation.

1.4.4.1 Rb and E2F

Using immunoprecipitation experiments in leukemia cell lysates, MDM2 was identified as a binding partner for the retinoblastoma protein (Rb). Like p53, the retinoblastoma gene is frequently mutated in human cancers, and the simultaneous inactivation of Rb protein is frequently observed in a variety of naturally occurring human tumors.

Rb is a nuclear phosphoprotein that arrests cells during the G1 phase of the cell cycle, by repressing the transcription of genes required for the G1/S transition (Weinberg, 1995). The tumor suppressor function of Rb was confirmed by its ability to inhibit the malignant phenotype when expressed in $Rb^{-/-}$ tumor cells (Huang *et al.*, 1988). A major cellular target of Rb is the E2F family of transcription factors (Dyson, 1998), which have binding sites in the promoters of many genes involved in cell cycle progression. Rb directly binds the transactivation domain of E2F, thereby blocking the ability of E2F to activate transcription (Helin *et al.*, 1993).

MDM2 has been reported to interact physically and functionally with Rb and, as with p53, inhibits Rb growth regulatory functions (Xiao *et al.*, 1995). Rb binds to the central region of MDM2 through its carboxyl-terminus (792-928 residues). Moreover, MDM2 can perturb Rb function in a p53-independent manner. MDM2 stimulates endogenous E2F transcriptional activity, which is normally bound and inhibited by Rb, through inhibition of Rb function, thus releasing cell cycle arrest at G1 phase (Sdek *et al.*, 2004). However, a direct interaction between MDM2 and E2F has been reported to stimulate the transactivation function of E2F in SAOS-2 cells, as E2F activity was elevated by MDM2 in cells expressing an MDM2 mutant defective in Rb binding (Martin *et al.*, 1995). It remains unclear whether the increased transactivation function of E2F in the presence of MDM2 is dependent on MDM2-Rb binding.

Interestingly, another report indicated that, besides being a downstream effector of MDM2, Rb could also act as a regulator for MDM2 (Hsieh *et al.*, 1999). Hsieh and colleagues discovered that Rb could overcome the anti-apoptotic function of MDM2 in p53-induced apoptosis. It was also shown that the C-terminus of Rb is essential and sufficient for overcoming the MDM2 anti-apoptotic effect. These results suggested that MDM2-Rb interaction may not only provide an opportunity for MDM2 to regulate Rb function, but also vice versa.

1.4.4.2 The CDK inhibitor $p21^{waf1/cip1}$

The role of cyclin-dependent kinase (CDK) inhibitor $p21^{waf1/cip1}$ is best defined in the p53 pathway. $p21^{waf1/cip1}$ is a direct transcriptional target of p53 and is strongly induced by DNA damage in cells expressing wild-type p53 (el Deiry *et al.*, 1994). It was initially discovered as a negative regulator of cell cycle progression, and mediates p53-dependent cell growth arrest and senescence (Roninson, 2002). The cell cycle inhibitory effects of $p21^{waf1/cip1}$ may be attributed to its ability to bind CDKs as well as proliferating cell nuclear antigen (PCNA), resulting in inhibition of cell cycle progression. Overexpression of $p21^{waf1/cip1}$ can result in cell cycle arrest in either G1 or G2, or in S phase (Ogryzko *et al.*, 1997;Niculescu, III *et al.*, 1998). Besides its CDK-binding activity, $p21^{waf1/cip1}$ interacts with a number of proteins directly involved in the regulation of transcription, growth, and DNA synthesis. In addition, upregulation of $p21^{waf1/cip1}$ protein levels can occur transcriptionally by p53-independent mechanisms (Sato *et al.*, 2002). A variety of transcription factors, including STATs, E2Fs, AP2, C/EBPα, C/EBPβ, and the homeobox transcription factor *gax*, can regulate $p21^{waf1/cip1}$ transcription through *cis*-acting elements in the $p21^{waf1/cip1}$ promoter (Gartel and Tyner, 1999).

MDM2 was found to promote $p21^{waf1/cip1}$ degradation via a proteasomal pathway (Jin *et al.*, 2003;Zhang *et al.*, 2004). This regulation is independent of p53 and is blocked by ARF through a subcellular separation mechanism. In these studies,

MDM2 was shown to interact physically with the carboxyl-terminus of $p21^{waf1/cip1}$ through its central domain. Moreover, experiments with deletion mutants of MDM2 indicated that degradation of $p21^{waf1/cip1}$ requires the central region of MDM2, while E3 ligase activity at the RING finger domain is not necessary for $p21^{waf1/cip1}$ turnover. This result is in agreement with a previous study which stated that turnover of $p21^{waf1/cip1}$ is independent of ubiquitination, although it is ubiquitinated in cells (Sheaff *et al.*, 2000). Furthermore, the nuclear localization sequence, but not nuclear export sequence, of MDM2 is important for its activity to promote $p21^{waf1/cip1}$ degradation, which is in line with the discovery that $p21^{waf1/cip1}$ degradation appears to occur solely in the nucleus (Sheaff *et al.*, 2000). Although the exact mechanism of exactly how MDM2 leads to $p21^{waf1/cip1}$ degradation still remains to be investigated, it is plausible that MDM2 may recruit the 26S proteasomal machinery to degrade $p21^{waf1/cip1}$, as MDM2 directly interacts with this protein. The identification of MDM2 as a regulator of $p21^{waf1/cip1}$ turnover provides an insight into my understanding of how MDM2 regulates the p53-independent pathway and the cell cycle.

1.5 Aim of thesis

The aim of this thesis is to investigate novel downstream target(s) of S6Ks, establishing a link between mTOR/S6K signalling and cell cycle regulation, and cell survival signalling. It has been mentioned in this chapter that S6K is an important player in the regulation of cell growth and cell cycle progression. The involvement of S6Ks in controlling protein synthesis, mainly at the stage of initiation, in response to various extracellular stimuli has been extensively studied in the last decade. Moreover, multiple lines of evidence link S6K signalling to G1/S transition and the rate of cell cycle progression. These include microinjection of antibodies to S6K in rat embryo fibroblasts which were able to abrogate serum-induced entry into S phase of the cell cycle, and the accelerated S phase entry by overexpressed S6K1 in U2OS cells. A downstream target of S6Ks directly linked to cell cycle regulation, however, has not yet been discovered.

The anti-apoptotic function of S6Ks was based on their ability to phosphorylate and inactivate pro-apoptotic protein BAD. In line with this statement, a report published by Pardo and colleagues indicated the involvement of S6K2 in the survival effect of FGF2 on small cell lung cancer cells. However, the molecular mechanisms by which S6K2 exert its effects on apoptosis still remain elusive.

In the present study, one of the primary objectives was to explore potential targets of S6Ks. Extensive bioinformatic and literature analysis allowed us to identify a panel of potential substrates for S6K activity. Further experimental studies clearly indicated that one of them, MDM2 is a true binding partner/substrate for S6K1 and S6K2. The finding that S6Ks phosphorylate MDM2 generated a further interest to determine the site(s) of phosphorylation on MDM2 by using mass spectrometry. The fact that another AGC kinase, PKB/Akt, was shown to be involved in the regulation of MDM2 prompted me to compare the contribution of both PKB/Akt and S6K to phosphorylation of MDM2 at serine 166 in response

to various extracellular stimuli. The experiments conducted in pursuit of these objectives are detailed in chapters three and four.

The discovery of an interaction between S6Ks and MDM2 and subsequent phosphorylation at serine 166 produced a second objective: to investigate the physiological role of this signalling event. This topic is addressed in chapter five, where the relevance of MDM2 regulation by S6Ks is investigated by different means, including confocal microscopy, transcriptional reporter assays, and siRNA technique.

CHAPTER TWO:

MATERIALS AND METHODS

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

Tissue culture media, phosphate-buffered saline (PBS), trypsin, G418, fetal bovine serum, and donor calf serum were obtained from Gibco. Chemicals were from Sigma, unless otherwise stated. Monoclonal anti-MDM2 antibody was from Oncogen. Polyclonal anti-phospho MDM2 (Ser166), anti-phospho-Rb (Ser807/811), anti-phospho S6 ribosomal protein (Ser235/236), anti-Akt/PKB, anti-phospho Akt/PKB (Ser473) and monoclonal anti-phospho S6K (Thr389) antibodies were from Cell Signalling. Monoclonal anti-p21 and polyclonal anti-p27, anti-E2F-1 antibodies were from Santa Cruz. Monoclonal anti-β-actin antibody was obtained from Sigma. The goat anti-mouse and anti-rabbit IgG HRP conjugated antibodies were purchased from Promega. Agarose conjugated protein A and protein G were from Amersham Biosciences. MG132, Rapamycin, and LY294002 were from Calbiochem. The source of other reagents and facilities are stated in the text as necessary.

The constructed mammalian and bacterial expressing human MDM2 plasmids were kindly provided by Prof. Karen Vousden (Cancer Research UK, Beatson Laboratories).

2.2 Methods

2.2.1 Nucleic acid manipulation

2.2.1.1 Bacterial strains and growth media

The following strain of bacteria was prepared as competent cells and used for transformation of plasmid DNA:

Escherichia coli XL-1 Blue cells. Genotype: supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac-F[proAB^{+}lacI9 lacZΔM15 Tn10(tet^{r})].

Luria Bertani (LB) broth and agar were purchased from Helena BioSciences. The LB media was prepared according the standard protocol by dissolving 20 g and 35 g of LB broth and LB agar respectively in 1L of ddH_2O. The prepared media was sterilized by autoclaving at 121^{0}C for 15 minutes.

Ampicillin and carbenicillin were prepared as 100 mg/ml stock solutions in ddH_2O and stored at -20^{0}C. Ampicillin and carbenicillin were used at a final concentration 100 μg/ml and 50 μg/ml respectively.

2.2.1.2 Preparation of competent *E. coli*

Competent *E. coli* cells were prepared by the following procedure: 5 ml of LB broth medium was inoculated with a single colony of *E. coli* and incubated overnight with shaking (200 rpm) at 37^{0}C. The overnight culture was diluted in 200 ml of fresh and pre-warmed LB medium in a 1 L flask and grown until it reached OD_{600nm}=0.4. The culture was cooled down by swirling the flask in a water-ice bath and bacteria were then pelleted by centrifugation at 1,000X g, 10 minutes, 4^{0}C. The bacteria pellet was resuspended in 1/40 of original volume of

ice-cold fresh LB medium; the total volume was measured. Following the addition of equal volume of ice-cold 2X TSS (Transfection Storage Solution, 10% (w/v) of PEG 3350, 5% DMSO, and 35 mM of $MgCl_2$ in LB broth with adjusted pH 6.5), the culture was mixed gently but thoroughly, and aliquoted on ice in precooled 0.5 ml tubes. The prepared competent cells were stored at -70^{0}C.

The method has transformation efficiency > 2X 10^7 cfu/µg of 3-5 kb of supercoiled plasmid DNA for XL1-blue strain.

2.2.1.3 Transformation of *E. coli*

Competent cells were thawed on ice and 100 µl cell suspension was mixed with 100 ng plasmid DNA. After 10 min incubation on ice, the cells were induced to take up the DNA by heat-shock at 42^{0}C for 2 min, cooled on ice for 10 min and allowed to recover in 500 µl of LB medium for 45 min at 37^{0}C in shaking incubator (225 rpm). The bacterial cells were then briefly centrifuged and pellet was resuspended in 100 µl of LB medium. Cell suspension was spread onto pre-warm LB agar plate containing the appropriate selective antibiotic and incubated overnight at 37^{0}C.

2.2.1.4 Purification of plasmid DNA

Plasmid DNA was purified using QIAGEN Plasmid Purification kit (QIAGEN), according to the manufacturer's directions. The QIAGEN plasmid purification protocol is based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to anion-exchange resin under appropriate low salt and pH conditions. Bacterial pallet from overnight shaker-culture of XL-1 Blue *E. coli* was resuspended in Resuspension buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A) and an equal volume of Lysis buffer (200 mM NaOH, 1%

Sodium n-dodecyl sulfate (SDS)) was added to the cell suspension. Following 5 minutes incubation at RT, the lysate was neutralized with 0.5 volume of chilled 3 M potassium acetate, pH 5.5 and incubated on ice for 5 min. Cellular debris was removed by centrifugation at 13,000 rpm for 10 minutes. The supernatant was applied to a QIAGEN-tip containing anion-exchange resin, pre-equilibrated with QBT buffer (50 mM MOPS, pH 7.0, 750 mM NaCl, 15% isopropanol, 0.15% Triton X-100), and allowed to move through by gravity flow. The resin was washed several times with Wash buffer (50 mM MOPS, pH 7.0, 1 M NaCl, 15% isopropanol) and DNA was eluted with an appropriate volume of Elution buffer (50 mM Tris-HCl, pH 8.5, 1.25 M NaCl, 15% isopropanol). The eluted plasmid DNA was desalted and concentrated by isopropanol precipitation. To precipitate the DNA, an equal volume of 100% isopropanol was added to the DNA solution and immediately centrifuged at 13000 rpm for 30 minutes. The DNA pellet was washed with 70% ethanol, air-dried for 5-10 minutes and redissolved in an appropriate volume of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and stored at -20^{0}C for long-term or 4^{0}C for short-term storage. The quantity and quality of the purified plasmid DNA was investigated as described in the section 2.2.1.5.

2.2.1.5 Quantification and qualification of plasmid DNA

To determine the concentration of plasmid DNA, a 1 µl aliquot was diluted in 1ml of TE buffer and OD_{260} was measured. An OD_{260} value of 1 was taken as being equal to a double stranded DNA concentration of 50 µg/ml. Quantity and quality of DNA samples were also checked by gel electrophoresis.

The electrophoretic mobility of DNA molecules depends on their size and the concentration of agarose gel used. 1% (w/v) gels were generally used, although 1.5% (w/v) gels were employed for analysis of DNA fragments less then 500 bp in size, and 0.8 % (w/v) gels were used for DNA fragments larger than 4 kb. The

appropriate weight of agarose was added to TAE buffer (40 mM Tris-Acetate, 1 mM EDTA) and heated to allow the agarose to dissolve. The solution was cooled down to approximately 60^0C and ethidium bromide was added to a final concentration of 1 μg/ml. The melted agarose solution was then poured into a mold and allowed to harden at RT. DNA samples were mixed with 6X loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (w/v) glycerol in water), loaded into the gel and plasmids were separated by electrophoresis in 1X TAE buffer at 90-100 V. Standard molecular weight markers (1 kb DNA ladder, Gibco) were electrophoresed alongside the samples. DNA was visualized and photographed under a long-wave UV light.

2.2.1.6 Double-stranded small interfering RNAs

The double-stranded small interfering RNAs molecules (siRNAs) against S6K1 (5'-GGACAUGGCAGGAGUGUUUTT-3'), S6K2 (5'-GGACCAAGAAGUCCAAGAGTT-3'), and PKB/Akt (5'-UGCCCUUCUACAACCAGGATT-3',), were manufactured by MWG Biotech AG based on published reports (Katome *et al.*, 2003;Harrington *et al.*, 2004).

2.2.2 Cell culture methodology

2.2.2.1 Cryopreservation of cells

In long-term, stocks of the various cell lines are kept in liquid nitrogen. To revive frozen cells, aliquots are removed from storage and thawed quickly at 37^0C in a water bath before transfer to a 50 ml tube containing prewarmed culture

medium. The medium was changed after 5 minutes centrifugation at the rate of 1,000X g to remove traces of the cryopreservation medium. The suspension of cells was transferred to a tissue culture dish or flask followed by incubation at 37^0C in a humidified incubator with 10% CO_2 for mammalian cells or at 27^0C for insect cells.

To freeze cells, following trypsinization of cell monolayers, 10 ml of complete medium is added. The cell suspension is transferred to a sterile 15ml tube and spun at 1,000 X g for 5 minutes at 4^0C. The supernatant is aspirated and the cell pellet resuspended in ice-cold freezing medium (culture medium containing 20% serum and 10% DMSO) to give a final cell density of $1X10^7$ cells/ml. 1 ml aliquots of the suspension are transferred to sterile cryovials, which are frozen slowly in a polystyrene box in a -80^0C freezer overnight. The following day, cells are transferred to liquid nitrogen for long-term storage.

2.2.2.2 Insect cell culture

2.2.2.2.1 Maintenance of insect Sf9 cells

The insect cell line from *spodoptera frugiperda* (Sf9) is maintained in IPL41 medium (Sigma) supplemented with 10% Fetal Calf Serum (insect cell culture tested), 2% yeastolate ultrafiltrate (Gibco), 1% lipid concentrate (Gibco), 1% fungizone (Gibco), 0.1% gentamycin (Gibco). Cells are grown in a 27^0C incubator. When cell monolayers reach confluence, cells are scraped gently using a cell scraper and then diluted in fresh medium in a new cell culture flask.

2.2.2.2.2 Baculoviral infection of Sf9 cells

For the production of recombinant S6K proteins, 2 X 10^7 cells were seeded in a 175 cm^2 flask and left to adhere for 3 hours. Then recombinant baculoviruses for S6K1 and S6K2 were added to the flask and the cells were cultured for further 48 hours. The medium was aspirated, the monolayer was rinsed briefly with PBS, and the cells were scraped from the flask gently to avoid disruption of the cells. The cell suspension was transferred in a 50 ml tube and further washed twice by addition of 20 ml PBS and centrifugation at 1,000 X g for 10 minutes at 4^0C. Then the cells were resuspended in 10 ml PBS, aliquoted by adding 1 ml in each of 1.5 ml tubes and spun down in a bench-top centrifuge at 2,000 X g for 5 minutes at 4^0C. The supernatant was aspirated and the cell pellets were snap frozen in liquid nitrogen and then stored in a -80^0C freezer.

2.2.2.3 Mammalian cell culture

2.2.2.3.1 Tissue culture media and maintenance of cell lines

Human embryonic kidney HEK 293 cells and human breast cancer MCF-7 cells were maintained at 37°C in humidified atmosphere containing 10% CO_2. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc), 2 mM L-glutamine, 50 U/ml penicillin and 0.25 μg/ml streptomycin. NIH 3T3 cells were grown in DMEM medium supplemented with 10% donor calf serum (DCS; Life Technologies, Inc.), 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. Human lung cancer H1299 cells and human prostate cancer PC-3 cells were maintained at 37°C in humidified atmosphere containing 5% CO_2. Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc), 2 mM L-glutamine, 50U/ml penicillin and

50 μg/ml streptomycin. Monolayer cells were split (1/6) for HEK 293, NIH3T3, and H1299 cells, or (1/3) for MCF-7 and PC-3 cells at 70-80% confluence. To dislodge cells medium was removed from tissue culture dish, cells were rinsed once with PBS and incubated in Trypsin-EDTA solution (Gibco BRL) for 2-4 minutes at room temperature. Cells were then suspended in complete DMEM or RPMI-1640 medium and split into 10-cm dishes.

Sub-culturing procedures were carried out in a laminar flow hood in a sterile environment using media/reagents that were all pre-warmed to 37^0C.

2.2.2.3.2 Transient transfection

Quality plasmid DNA constructs for transfection were prepared as described in section 2.2.1.2 and DNA was re-precipitated under sterile conditions. Cells were seeded at 1.0×10^6 per 60 mm dish 16 hours prior to transfection. Transient transfection was performed with 2.5-10 μg of total DNA using LipofectAMINE reagent (Life Technologies, Inc.) according to the manufacturer's recommendations. For each transfection plasmid DNA and 10 μl of LipofectAMINE reagent were separately diluted in 100 μl of Opti-MEM I Reduced Serum Medium (Gibco BRL). Mixtures were incubated for 10 minutes at RT, combined and then left at RT for the next 30 minutes to allow the formation of DNA-liposome complexes. During this period of time cells were rinsed once with 2 ml of serum-free DMEM medium and 2 ml of Opti-MEM I medium was added to the dish. DNA-liposome complex solution was overlaid onto the cells and dishes were incubated for 3-5 hours at 37°C in a CO_2 incubator. Following incubation the medium was replaced with complete DMEM medium and transfected cells were grown for the next 24-48 hours. Recombinant protein expression was analysed to optimize transfection conditions for individual plasmid DNA. For PC-3 and HEK293 cell lines, cells were seeded at 2.0×10^5/35-mm dish 16 hours prior to transfection, followed by transfection

procedure with 2-4 μg of total DNA and 5-10 μl of ExGen 500 *in vitro* transfection reagent (Fermentas) according to the manufacturer's recommendations. For each transfection, plasmid DNA was diluted in 100 μl of sterile 150 mM NaCl and vortex for 10 seconds. The ExGen 500 reagent was added to the DNA mixture before vortex for another 10 seconds. The DNA/ExGen 500 mixture was incubated at room temperature for 10 minutes before adding into the dish with cells. Cells were grown for 24-48 hours giving time for the expression of the recombinant proteins.

2.2.2.3.3 Transient transfection of double-stranded siRNAs

To transient transfect siRNAs into cells, HEK 293 cells and LipofectAMINE™ 2000 transfection reagent (Invitrogen) were used. Cells were seeded at 25×10^4 cells per 60 mm culture dish one day before transfection. For each transfection sample, 400 hundred pico-mol of siRNA and 10 μl of LipofectAMINE™ 2000 reagent were separately diluted in 250 μl of Opti-MEM I Reduced Serum Medium (Gibco BRL). Mixtures were incubated at RT for 10 minutes, combined and left at RT for the next 30 minutes to allow the formation of siRNA-liposome complexes. During this period of time, the growth medium of cell was changed into antibiotic-free DMEM medium (2.5 ml per 60 mm dish). After the formation of siRNA-liposome complexes, the siRNA- LipofectAMINE™ 2000 mixture was overlaid onto the cells and dishes were incubated at 37°C in a CO_2 incubator. Five hours after transfection, fresh antibiotic-free DMEM medium were added to the cells (2.5 ml per 60 mm dish). The transfected cells were grown in the antibiotic-free DMEM for the next 4 days before harvesting.

2.2.2.3.4 Generation of stable cell lines

To generate stable cell lines, PC-3 cells and ExGen 500 *in vitro* transfection

reagent was used. In this system, the pcDNA1/S6Kα ΔNΔC-Flag, pcDNA1/S6Kβ ΔNΔC-Flag, and pcDNA1/mTOR RR-Flag do not have the G418 resistant gene, while the pcDNA3 empty vector has the neomycin resistant gene as a selection marker and was mixed with the non-selectable constructed plasmids for transfection. PC-3 cells were seeded and transfected as described in section 2.2.2.3.2, except of the presence of non-selectable and selectable plasmid DNA in the ratio of 10:1. Cells were cultured in the growing medium for 48 hours, followed by splitting 1/10 into complete medium containing 750 μg/ml of G418 (neomycin, Gibco). Selective medium was changed every 3 days and cell survival was monitored over the period of two weeks to identify colonies that had integrated a gene construct. Discrete colonies were trypsinised and collected into new plates, maintained in the medium containing 750 μg/ml of G418 as well as frozen in liquid nitrogen as long-term stocks. The expression of the recombinant proteins was monitored by Western Blotting with specific antibodies.

2.2.2.3.5 In vivo labeling cells with [^{33}P] orthophosphate

MCF-7 cells were grown in 60 mm dishes with complete phenol red-free DMEM medium to approximately 60-70% confluency followed by starvation in phenol red-free DMEM medium with 0.1% serum for 18 hours. Cells were washed twice with sodium phosphate-free DMEM medium and incubated in this medium for 1 hour at 37°C. After incubation the medium was removed and fresh sodium phosphate free DMEM medium supplemented with radioactive [^{33}P] Orthophosphate (50 μCi/ml, Amersham Pharmacia Biotech) and MG132 (10 μM, Sigma) was added to the cells. Cells were then incubated for 6 hours (short-term labelling) or 18 hours (long-term labelling) at 37°C. Cells were treated with LY294002 or Rapamycin 30 minutes before IGF-1 stimulation. After 30 minutes of IGF-1 stimulation, cells were washed twice with ice-cold PBS and lysed on ice in appropriate buffer for further studies.

2.2.2.3.6 Starvation and stimulation of cells

For standard serum starvation, cell monolayer was washed 2 times with pre-warmed PBS and the medium without serum was added to the plates. Starvation was proceeded for the time according to different cell types. For amino acid starvation, cells were starved in serum-free medium for 16 hours, followed by additional starving in 1X PBS supplemented with 0.1 g/L of $CaCl_2$ for another 2 hours. Inhibitors may be added during this period of time before stimulation with certain concentration depending of each experiments.

To stimulate cells with serum, the serum-free medium was replaced with complete growth medium and cells were incubated for the time as indicated. For amino acid stimulation, concentrated essential amino acid mix (Gibco) was added to the plates and incubated for 30 minutes before lysing.

2.2.3 Protein purification and analysis

2.2.3.1 Purification of GST-fusion recombinant proteins

pGEX4T-1 plasmids containing sequences for the full-length or various fragments of human MDM2 were used to transform competent *E.coi.* XL 1-Blue cells. After selection on ampicillin-containing agar plates, a single colony was used to inoculate a 100 ml starter culture in LB broth medium containing 100 μg/ml ampicillin. The culture was grown overnight with shaking at 37°C and the next day used to inoculate in 1000 ml of LB broth medium supplemented with 100 μg/ml ampicillin. The suspension was grown at 37°C with shaking until it reached an OD_{600nm}=0.6. Expression of recombinant proteins were induced by

adding IPTG at a final concentration of 0.4 mM, and followed by incubation for further 4 hours with shaking at 25°C. Bacteria cells were pelleted at 3,500 rpm, 4°C, for 20 minutes and washed once with 40 ml ice-cold PBS. Pelleted cells were resuspended in 10 volumes (ml/g cell pellet) ice-cold lysis buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 50 mM NaF, 50 mM NaCl, and 1% (v/v) Triton X-100 supplemented with 1 mM PMSF) and kept on ice for 20 minutes. Following 20 minutes incubation, the suspensions were sonicated on ice by applying three rounds of 20 seconds pulses with 20 seconds intervals between pulses. The lysates were centrifuged at 18,000 rpm for 30 minutes at 4°C to remove the insoluble fraction. Proteins were isolated from the lysates by rotating with pre-washed Glutathione-sepharose 4B (Pharmacia) in 15 ml Falcon tubes at 4°C for two hours. Sepharose beads with bound GST fusion proteins were washed four times in lysis buffer, followed by two washes with 50 mM Tris-HCl, pH 7.5. The bound GST fusion proteins were eluted by incubating with 3 ml of 20 mM Glutathione in 100 mM Tris-HCl, pH 8.0, 150 mM NaCl. The elution was repeated for another two times. Subsequently, in order to remove free glutathione, the mixtures of eluted proteins were dialyzed against the buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM DTT for 12 hours, followed by another dialysis against buffer with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM DTT in 50% glycerol. The dialyzed proteins were then stored at -20°C.

2.2.3.2 Preparation of mammalian cell extracts

To extract cellular protein content, cells were washed two times with ice-cold PBS and extracted with pre-chilled extraction buffer (EB) containing 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 50 mM NaF, and 150 mM NaCl, and also 1% (v/v) Triton X-100. Total cell lysates were incubated on ice for 30 minutes and then centrifuged at 13000 rpm for 20 minutes at 4°C to pellet the insoluble cell debris. The supernatant was then removed and used for further analysis, including SDS-PAGE and immunoblotting, immunoprecipitation and protein kinase assay.

In the case of detecting protein-protein interactions, HEK 293, MCF7, U2OS, and NIH 3T3 cells were washed twice with ice-cold PBS and extracted with pre-chilled low salt association buffer (LSAB) containing 100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 % v/v Nonidet P-40 (NP-40), 1 mM sodium orthovanadate, and protease inhibitor cocktail (Roche). Total cell extracts were incubated on ice for 30 minutes and clear lysate suspensions were prepared by centrifugation of the total cell lysates at 13,000 rpm for 20 minutes at 4°C.

Total protein concentration of the lysates was determined by Bradford protein assay (section 2.2.3.3).

2.2.3.3 Estimating protein concentration

To estimate protein concentration in cell lysates, the colorimetric method, involving Coomassie Protein Reagent (Pierce), was used. The method is based on the absorbance shift from 465 to 595 nm, which occurs when Coomassie brilliant blue G-250 binds to proteins in an acidic solution. 0.5 ml of Coomassie Protein Reagent was diluted in 0.5 ml of ddH_2O and 1 µl of cell lysate was added to the mixture. Upon 30 minutes incubation at RT the absorbance was measured at OD_{595} and compared with a blank control. The protein concentration was then determined by comparison of absorbance values with a bovine serum albumin (BSA) standard curve.

2.2.3.4 SDS-PAGE protein electrophoresis

Cellular proteins were separated based on their molecular weight (MW) using the discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) system as described by Laemmli (Laemmli, 1970). In a discontinuous system, a non-restrictive large pore gel, called a stacking gel, is layered on top of a

separating gel, called a resolving gel. The proteins run through the stacking gel as tight bands and are only separated when they migrate through the resolving gel, which is characterized by higher pH and acrylamide concentration. The percentage of acrylamide in resolving gel may vary depending on the range of separation desired. 7.5%-12.5% acrylamide concentration was generally used. To prepare the resolving gel, 30% acrylamide stock solution (acrylamide:N,N'-methylene bis-acrylamide 37.5:1) was diluted with appropriate volume of ddH_2O and 1.5 mM solution of Tris-HCl, pH 8.8 and 10% SDS solution were added to the final concentration of 375 mM and 0.1% (w/v) respectively. Polymerization was initiated by the addition of ammonium persulfate (0.05% (w/v)) and TEMED (0.005% (v/v)). The gel mixture was then promptly poured into glass plate assembly and overlaid with water-saturated butanol to ensure a flat surface and to exclude air. After polymerization butanol was removed and gel surface was washed with water. The stacking gel mixture (4.5% (w/v) acrylamide) was prepared in the same way in 0.125 M Tris-HCl buffer, pH 6.7, poured onto top of set resolving gel and left to polymerize with spacer comb inserted into the mixture. Following polymerization, the comb was removed and created wells were flushed and filled with SDS electrophoresis buffer (25 mM Tris-HCl, pH 8.3, 195 mM glycine, 0.1% (w/v) SDS). 1 part of cell lysates or protein solutions was mixed with 1 part of 2× SDS-PAGE sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.2 M DTT, 0.1% Bromophenol Blue), heated for 5 minutes at 95°C and then samples were loaded under buffer into stacking gel wells. Electrophoresis was run in electrophoresis buffer at a fixed current of 20-30 mA per gel until the dye front reached the end of the gel. Visualization and analysis of separated proteins were performed as described in sections 2.2.3.5 and 2.2.3.6.

2.2.3.5 Visualization of proteins

2.2.3.5.1 Coomassie Blue staining

Following electrophoresis, some gels were stained for the detection of proteins by soaking in Coomassie Blue stain (0.2% (w/v) Coomassie brilliant blue R-250, 45% (v/v) methanol and 10% (v/v) acetic acid) for 20 minutes, followed by distaining in 20% (v/v) methanol and 5% (v/v) acetic acid with agitation. The gel was then dried under vacuum at 80°C for 1 hour. Coomassie brilliant blue binds to proteins stoichiometrically, so this staining method is preferable when relative amounts of protein are to be determined by densitometry.

2.2.3.5.2 Silver staining

Silver staining is one of the most sensitive nonradioactive methods of protein detection in gel which allows analysing nanogram quantities of protein. In this study, I have used the method described by Shevchenko *et al.* (Shevchenko *et al.*, 1996). After electrophoresis, the polyacrylamide gel was fixed for 20 minutes in solution containing 50% methanol and 5% acetic acid, rinsed with 50% methanol for 10 minutes and ddH_2O for another 10 minutes. Sensitization step was carried out for 1 minute in 0.02% $Na_2S_2O_3$ solution, followed by two brief washing with ddH_2O. The gel was then stained in pre-chilled 0.1% $AgNO_3$ solution for 20 minutes at 4°C and developed in 2% Na_2CO_3/0.04% formalin solution to the desired degree. Development was stopped by incubation of the gel in 5% acetic acid for 10 minutes. Gels were stored in 1% acetic acid at 4°C or dried.

2.2.3.5.3 Autoradiographic exposure of acrylamide gel

This method was used to detect radioactively labeled proteins in acrylamide gel. Following SDS-PAGE separation of proteins, the acrylamide gel was stained with Coomassie Blue as described in section 2.2.3.5.1. The stained gel was then

placed on a piece of Whatmann 3 MM filter paper, dried for 30 minutes, and exposed to an X-ray film (Fuji) or a phosphor screen. The images of radio-labeled protein bands were acquired using a phosphoimager (Bio-Rad) and analysed with manufacturer provided software.

2.2.3.6 Immunoblot analysis

2.2.3.6.1 Wet transfer of proteins

Proteins separated by SDS-PAGE as described in section 2.2.3.4 were transferred onto a nitrocellulose membrane or Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore). The PVDF membrane was pre-wetted in methanol briefly and rinsed in water for two minutes to increase the hydrophilic property. Both membrane and the gel were equilibrated in transfer buffer (190 mM glycine, 25 mM Tris Base and 20% (v/v) methanol) for approximately 5 minutes. The gel was then placed on top of a nitrocellulose membrane or methanol-soaked PVDF membrane, both of which were immersed in transfer buffer, and sandwiched in a compression cassette between several layers of pre-wetted Whatmann 3 MM filter paper. The transfer was performed in Trans-Blot^{TM} electrophoretic transfer cell, according to the manufacturer's instructions (Bio-Rad) at 60 V for 120 minutes.

2.2.3.6.2 Western blotting and ECL immunodetection

This method, developed by Amersham, was subsequently used for all antibody detection because of the speed of the reaction and the exclusion of radioactivity. ECL is a light emitting, non-radioactive method for the detection of immobilized specific antigens with antibodies conjugated to horseradish peroxidase. The

system utilises a chemiluminescent reaction which takes place when the cyclic diacylhydazide luminol is oxidised in the presence of the hydrogen peroxide (H_2O_2). Following oxidation, the luminol is in an excited state, which decays to the ground state via a light emitting pathway.

After wet transfer procedure, the membrane was incubated in blocking buffer (5% non-fat dry milk, 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.05% Tween 20) for 1 hour at RT to block non-specific binding sites. The membrane was then incubated with the primary antibodies in minimal volume of blocking buffer at the appropriate dilution (1/200-1/2,000) for 1 hour at RT or overnight at 4°C. Excess antibodies were then removed by washing in blocking buffer. At this step membrane was rinsed once in blocking buffer and then washed once for 15 minutes and twice for 5 minutes with fresh changes of the buffer at RT. After washing the membrane, a species-specific horseradish peroxidase-conjugated secondary antibody (Promega) was then applied in a minimal volume of blocking buffer for 1 hour at RT at a dilution 1/5,000. Following incubation with secondary antibodies the membrane was rinsed once in blocking buffer and then washed five times in TBS-T buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) for 5 minutes at RT. Immunoreactive proteins were detected by enhanced chemiluminescence. Equal volumes of ECL detection solution 1 and solution 2 (Amersham) were mixed and added to the membrane. The reaction was allowed to proceed for 1 minute at RT and excess of horseradish peroxidase substrate was removed. The membrane was dried on tissue, wrapped in SaranWrap, and exposed to X-ray film for various period of time or scanned by fluoroimager (Bio-Rad). The signal was quantified using manufacturer provided software.

2.2.3.6.3 Stripping and reprobing

Immunoblot membrane may be stripped of bound antibodies and reprobed several times. Membranes should be stored wet wrapped in SaranWrap in a refrigerator

(2-8°C) after each immunodetection. The membrane was pre-wetted in methanol and submerged in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) incubated at 50°C for 30 minutes with occasional agitation. The membrane was then washed in TBS-T buffer several times for 10 minutes at RT, blocked for 1 hour at RT in blocking buffer containing 5% fat-free milk and immunoblotted as described above.

2.2.3.7 Immunoprecipitation

Cells monolayers were rinsed twice with ice-cold PBS and lysed in LSAB buffer as described in section 2.2.3.2. After centrifugation, the extracts were incubated with appropriate antibodies (1 μg of antibody per 1 mg of total protein) at 4°C on a rotating wheel for 2 hours. 20 μl of 50% protein G-Sepharose suspension was added to each sample to bind antibody-protein complexes and the incubation continued for a further 1 hour. Following the incubation with protein G-Sepharose, the immune complexes were then pelleted by low speed centrifugation (2,500 rpm) and washed 4 times with ice-cold lysis buffer. 20 μl of 2X SDS-PAGE sample buffer was added to the immune complexes, which were then boiled at 95°C for 5 minutes and separated by gel electrophoresis.

2.2.3.8 Immune complex ribosomal protein S6 kinase assay

Endogenous MDM2 protein was immunoprecipitated from MCF-7 cell lysates with anti-MDM2 antibody immobilized on protein G-Sepharose beads. The immune complexes were washed three times with lysis buffer, followed by a single wash with kinase assay buffer (50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiotreitol (DTT), 10 mM β-glycerophosphate). The kinase reaction was initiated by resuspending the beads in 25 μl of kinase assay buffer supplemented with 1 μM protein kinase A inhibitor (PKI, Calbiochem), 50 μM ATP, 5 μCi of

[γ-^{32}P]ATP (Amersham), and 20 μg of 80S ribosomes, isolated from rat liver (Thomas *et al.*, 1978). The reaction was carried out at 30°C for 30 minutes and terminated by addition of 5 × SDS-PAGE sample buffer and boiling for 5 minutes. Samples were subjected to 10% SDS-polyacrylamide gel electrophoresis, and the amount of ^{32}P incorporated into S6 protein was assessed by autoradiography and quantified by phosphoimager (Bio-Rad).

2.2.3.9 *In vitro* phosphorylation of MDM2 by S6K

The GST-fusion recombinant MDM2 proteins were purified as described in section 2.2.3.1, and used as the substrates for the *in vitro* S6 kinase assay alone with S6 protein as a positive control. One μg of each GST-MDM2 fusion proteins was mixed with 0.5 μg of S6K1 or S6K2 recombinant protein. The reaction was initiated by adding 35 μl of kinase assay buffer as described in section 2.2.3.8 with the supplement of 1 μM protein kinase A inhibitor (PKI, Calbiochem), 50 μM ATP, and 5 μCi of [γ-^{32}P]ATP (Amersham). The reaction was incubated at 30°C for 30 minutes and terminated by addition of 5 × SDS-PAGE sample buffer and boiling for 5 minutes. Samples were separated and analysed as described above.

2.2.3.10 Immunofluorescent staining and confocal microscopy

Cells were plated onto poly-L-lysine coated coverslips in 4-well dishes at a density of 2-3 X 10^4 cells per well and cultured overnight. The cells were transfected with 0.5 μg of expression vectors containing various MDM2 constructs. Twenty four hours post-transfection cells were starved in serum-free DMEM medium for 24 hours and then stimulated with 10% serum for 1 hour. Following two times wash with PBS, the stimulation was terminated by fixation with 250-500 μl of 4% formaldehyde in PBS for 20 minutes, and permeabilized

with 0.2 % Triton-X100 in PBS for 5 minutes at RT. Non-specific binding was blocked by incubation with 0.5 % bovine serum albumin in PBS for 30 minutes at RT. The cells were then incubated with appropriate antibodies for 2 hours at RT. After extensive washing with PBS, the samples were incubated for 45 min with goat fluorescein isothiocyanate-conjugated (FITC) anti-mouse or anti-rabbit antibodies (1:200 dilution). Finally, the coverslips were extensively rinsed with PBS, air-dried and mounted onto microscope slides using maviol mounting medium. Immunofluorescent staining was analysed using Laser Scanning Microscope LSM51D (Zeiss, Germany), using 40×/1.30 oil Plan-Neofluar immersion objective (Zeiss, Germany). As a source of illumination for confocal microscopy Krypton/Argon laser, which emits at three wavelengths 488, 568 and 647 nm, was used.

2.2.4 Production of anti-phospho Ser166 MDM2 antibody

2.2.4.1 Generation of rabbit antisera

Polyclonal antiserum that recognizes specific phosphorylation at the sites of Ser166 and Ser186 on MDM2 was raised by immunizing rabbits with synthetic phosphopeptides: CTSSRRRAIpS^{166}ETEEN (MDM2 pSer166) and CGERQRKRHKpS^{186}DSIS (MDM2 pSer186).

2.2.4.2 Affinity purification of phosphor specific antibody

The antibodies generated were affinity-purified using antigenic peptides coupled to Actigel (Sterogene). Five mg of the synthetic peptide were dissolved in 300 µl of coupling buffer (100 mM phosphate buffer, pH 7.8). Small portions of 5 M

NaOH and/or 100% DMSO were added into the mixture to optimise peptide solubilisation. 0.5 ml of 50% Actigel beads, pre-washed several times with coupling buffer, were mixed with 250 µl of solubilised peptide. Coupling of the peptide to Actigel was initiated by adding 1 M $NaCNBH_3$ (1/10 of final reaction volume), and carried out on a wheel for 4-6 hours at 4^0C. After coupling, the beads were washed twice in buffer containing 100 mM Tris-HCl, pH 8.0 and 500 mM NaCl and once in 100 mM Tris-HCl, pH 8.0. To block uncoupled sites, the Actigel beads were finally incubated with 100 mM Tris-HCl, pH 8.0 for 2-4 hours and then stored at 4^0C in the presence of 0.02% (w/v) sodium azide.

Polyclonal antiserum was centrifuged at 15,000 rpm at 4^0C for 10 minutes and loaded into a column prepared with peptide coupled-Actigel. The column was allowed to empty by gravity flow and then washed extensively with PBS. Bound antibody was eluted with 0.1 M glycine, pH 3.0 and collected as 1 ml fractions into tubes containing 100 µl of 1 M Tris-HCl, pH 8.0. Protein concentration was measured as described in section 2.2.3.3. To remove IgGs, recognizing non-phosphorylated MDM2 protein, peak fractions were combined and loaded onto Actigel column containing beads coupled with MDM2 (166 or 186) non-phosphorylated peptides. The protein fraction was combined, dialysed twice against PBS and once against 50% glycerol/PBS. The purified antibodies were stored at -20^0C.

Affinity purified antibodies were screened for antigen reactivity by immunoblot analysis (section 2.2.3.6).

2.2.5 Analysis of cell cycle and cell growth

2.2.5.1 Cell cycle analysis by FACS

Cell monolayer was harvested in the appropriate manner and collected in a 15 ml Falcon tube. Following the centrifugation of cells at RT, 1,000X g, 5 minutes, the pellet was washed once with 1X PBS and fixed with 2ml of 70% ethanol while vortexing. Fixation was proceeded for at least 30 minutes at 4^0C. Another two wash was applied on the fixed cells before staining the DNA content with 400µl of propidium iodide (PI, 50 µg/ml) with 20 µg/ml RNase for 15 minutes at RT. Analysis of the DNA content was performed by flow cytometry (FACS Calibur, Becton Dickinson) measuring PI fluorescence above 600 nm with at least 50,000 cells.

2.2.5.2 Analysis of cell growth

Casy model TT electronic cell counter (CASY-Technology) was used to determine the growth curve of certain cell lines. Equal amount of cells were seeded in 60 mm dishes and grown overnight. Cells were harvested by standard sub-culturing procedure described in section 2.2.2.3.1 according to the setting of different time points and collected in 1.5 ml tubes. One hundred µl of the cell suspension was mixed with 10 ml of Casy buffer provided by the manufacture and the number of the cells was measured by the Casy cell counter with appropriate settings.

2.2.6 Reporter assay

2.2.6.1 Principle of reporter system

Genetic reporter systems have developed into an essential tool for examining

regulatory promoter and enhancer sequences as well as testing the activity of transcription factors. In most cases, the element under investigation (promoter, enhancer) is cloned together with the reporter gene in an expression vector, which is subsequently used to transfect cells. Quantification of the reporter indirectly provides information on the transcription activity of the element under investigation. Quantification can take place by detecting the corresponding RNA, the reporter protein, or by measuring the enzyme activity of the reporter protein. When the reporter system is selected, care must be taken to ensure that the reporter gene does not influence the physiology of the transfected cells and that the gene is not already endogenously expressed in the examined cells.

Reporter systems are also often used as a standard to compare the transfection efficiency of different transfection experiments. In this case, the control reporter system usually contains a constitutive promoter and a reporter gene, which is different to the reporter gene used by the element under examination.

2.2.6.2 Establishment of reporter assay

The Mercury™ Pathway Profiling Luciferase System containing pE2F-TA-Luc, pRb-TA-Luc, pp53-TA-Luc, and pMyc-TA-Luc reporter plasmids was obtained from BD Biosciences, and the p53-CAT reporter plasmid was kindly provided by Dr. M. Tarunina (Ludwig Institute for Cancer Reasearch, University College London, UK).

Cells were seeded in 6-well plates one day before transfection, and standard transfection procedure was carried out with 2 μg of total plasmid DNA and 6 μl of ExGEN 500 transfection reagent as described in section 2.2.2.3.2. Twenty-four hours after transfection, cells were lysed in the appropriate manner according to the method adapted to the reporter assay.

2.2.6.3 Measuring reporter activities

2.2.6.3.1 Chloramphenicol Acetyltransferase (CAT)

Chloramphenicol acetyltransferase (CAT), encoded by a bacterial drug-resistance gene, inactivates chloramphenicol by acetylating the drug at one or both of its two hydroxyl groups. This gene is not found in eukaryotes, and therefore eukaryotic cells contain no background of CAT activity. This characteristic, along with the ease and sensitivity of the assay for CAT activity, has made the CAT gene one of the first reporter genes used for studies of mammalian gene expression. CAT activity may be monitored by two alternative methods in the CAT Enzyme Assay System. The most rapid, sensitive and convenient of these assays is based on liquid scintillation counting (LSC) of CAT reaction products. CAT activity can also be analyzed by using thin-layer chromatography (TLC). This method is more time-consuming than the LSC assay but allows visual confirmation of the data.

For CAT transient expression assays, cell extracts are typically prepared 48-72 hours post-transfection. After washing cell monolayer twice with warm 1X PBS, 0.5-0.6 ml of TEN buffer (40 mM Tri-HCL, pH 7.5, 1 mM EDTA, pH 8.0, 150 mM NaCl) was added into the plates and the cells with TEN buffer were incubated at RT for 5 minutes. Cells were collected in 1.5 ml eppendorf tubes and spun down at 13,000 rpm, 4^0C, for 2 minutes. The supernatant was discarded and the cell pellet was resuspended in 60 µl of 100 mM Tris-HCl, pH 7.8. The samples were proceeded in freeze/thaw cycle in a dry ice/100% ethanol bath for 4 times before incubating them at 60^0C for 10 minutes. Following maximum speed centrifugation for 3 minutes, the clear lysates were transferred into new tubes. To perform CAT assay, 30 µl of the lysates were aliquoted in scintillation tubes with 20 µl of 100 mM Tris-HCl, pH 7.8 to make the final

volume up to 50 µl. Twenty-five µl of 1 M Tris-HCl, pH 7.8, 5 µl of 50mM chroramphenicol, 3 µl of ^{3}H-labbled Butyryl Coenzyme A, and 166 µl of ddH_2O were added in the tubes, followed by the adding 3.5 ml of counting scintillant. Samples were incubated in the water bath at 37^{0}C for 1-3 hours and the activity of CAT was measured with a scintillation counter. All the measurements were standardized by analysing protein concentration of each sample.

2.2.6.3.2 Firefly Luciferase

The enzyme from the North American firefly (Photinus pyralis) catalyzes a bioluminescence reaction. In the luciferase assay, the lysates of transfected cells are incubated with luciferin, molecular oxygen, ATP and Mg^{2+}. In the following reaction, the luciferase catalyzes the oxidation of luciferin in oxyluciferin and CO2. In this reaction, light with a wavelength of 562 nm is emitted, which then fades rapidly. The light emitted can be measured in a luminometer or in a liquid-scintillation counter. Light emission is proportional to the amount of luciferase in the lysate, thus enabling the indirect quantification of the transfection rate of the reporter gene. The sensitivity of the luciferase assay is further increased by adding co-enzyme A to the reaction preparation, rendering it 10–20 times more sensitive than the CAT assay.

The luciferase reporter assay kit was purchased from BD Biosciences. Cells were washed two times in cold PBS and lysed with 200 µl of 1X Cell Lysis Buffer as standard procedure described previously. The clear cell lysates were prepared by centrifugation at 13,000 rpm, 40C, for 1 minute, and allowed to reach RT. For measuring the luciferase activity, 25 µl of the lysates were aliquoted into a flat-bottom 96-well plate. Following the addition of 50 µl of Solution A and B, which are provided with the kit and were pre-warmed to RT, the reactions were proceeded directly to a scintillation counter to measure the light emission in each well. Each result was standardized by the protein concentration of each sample.

CHAPTER THREE:

THE ANALYSIS OF INTERACTION BETWEEN S6K AND MDM2 *IN VITRO* AND *IN VIVO*

CHAPTER THREE

THE ANALYSIS OF INTERACTION BETWEEN S6K AND MDM2 *IN VITRO* AND *IN VIVO*

3.1 Introduction

Protein-protein interactions are crucial for the formation of multienzyme complexes regulating signal transduction pathways. Both isoforms of S6K were shown to transduce signalling information downstream of PI3K and mTOR pathways in response to mitogenic stimuli and nutrient sufficiency. It is assumed that S6Ks receive signalling information from upstream regulators by integrating into multienzyme signal transduction complexes. In contrast to other signalling molecules, S6 kinases do not possess canonical protein-protein interaction domains, such as SH2 and SH3 pathways or domains which can bring them in complex with membrane's phospholipids (PH or FIVE) or nucleic acids (HLH, Zn-finger etc). However, the presence of a proline-rich motif in S6K2 or a PDZ-binding motif in S6K1 may drive specific signalling interactions with molecules possessing SH3 and PDZ domains respectively. In addition, both S6Ks contain a TOR signalling sequence (TOS), which is a recognition motif for the substrate-presenting protein raptor. Interaction with raptor is crucial for mTOR-mediated phosphorylation of S6K in the hydrophobic pocket site (Thr389 in S6K1 and Thr401 in S6K2).

So far, very few S6K binding partners have been identified. These include proteins with diverse cellular functions, including protein kinases PDK1 and PKC; protein phosphatases PP2A and PP1; small GTPases Rac and cdc42; cytoskeletal protein neurabin and mRNA processing protein SKAR (Westphal *et al.*, 1999;Martin *et al.*, 2001;Valovka *et al.*, 2003;Richardson *et al.*, 2004;Nemazanyy *et al.*, 2004;Sarrouilhe *et al.*, 2006). In order to identify novel binding partners

for S6K, several approaches have been applied in out laboratory, including the yeast two-hybrid screening, and affinity purification followed by mass spectrometry. These studies led to the identification of CoA synthase, protein kinases PKC and CK2 as novel binding partners for S6K1 and S6K2 (Valovka *et al.*, 2003;Nemazanyy *et al.*, 2004;Panasyuk *et al.*, 2006). In order to search for novel binding molecules and potential substrates for S6K, I have applied a bioinformatic approach which allows the identification of potential binding partners/substrates based on the sequence specificity of the substrate phosphorylation motif. This analysis, combined with detailed literature searches, allowed me to select a panel of cellular proteins as potential candidates for interaction and phosphorylation by S6Ks. Further experimental studies clearly indicated that MDM2 in particular, is a true binding partner for S6K1 and S6K2.

In this chapter, interaction specificity between S6K1/2 and MDM2 is unequivocally demonstrated with the use of recombinant proteins (GST pull-down assay), and by co-immunoprecipitation of transiently overexpressed endogenous proteins. Moreover, I found that S6K/MDM2 interaction is induced in response to serum of growth factor stimulation.

3.2 Results

3.2.1 Bioinformatic search for potential binding partners / substrates for S6K

Previous studies from various laboratories showed that S6K preferentially phosphorylate substrates with following sequence motif: RXRXXS/T, where X is any amino acid (Alessi *et al.*, 1996b;Shah and Hunter, 2006). Taking this into account, I performed an extensive analysis of SWISS-PROT database with the S6K substrate phosphorylation motif RXRXXS/T. These searches yielded several hundred cellular proteins which possessed one or several S6K phosphorylation motifs. Then, I selected those proteins whose cellular functions are known and overlap with that mediated by S6K, including the regulation of ribosomal biogenesis, initiation of protein synthesis, energy metabolism and cell survival. To narrow down the list (over 40 proteins) even further, I performed literature analysis (PubMed database) for studies which demonstrate indirectly the link between selected proteins and mTOR/S6K signalling (the sensitivity to rapamycin, activation/inhibition by growth factor and nutrient signalling were among the search criteria). Finally, I selected 10 potential candidates which were considered as the best hits for further characterization. These included translation initiation factor eIF-2B, DNA polymerase subunit A, transcription factors FOS-B and Jun-B, Histone deacetylases 4 and 5, MDM2, Rb binding protein 2 and 5, diacylglycerol kinase, caspases 7-9 etc. Among selected proteins, MDM2 was the frontrunner since: a) MDM2 possesses two potential S6K phosphorylation motifs, located near nuclear localization and nuclear export sequences; b) the function and subcellular localization of MDM2 is regulated by multiple phosphorylations mediated by various signal transduction pathways, including PI3K/PKB signalling; c) MDM2 has been implicated in ribosomal biogenesis through regulation of RNA polymerase 1 and the association with ribosomal proteins (such as L5); d) it is localized to nucleoli where ribosomes are

formed; e) MDM2 is a key player in the regulation of the cell cycle via p53 signalling pathway. Numerous studies have implicated mTOR/S6K signalling in the cell cycle control, especially the G1 to S transition; f) some reports, although controversial, indicate that the of MDM2/p53 signalling is sensitive to rapamycin; g) the function of p53 was found to be regulated by mTOR phosphorylation at Ser15.

Taking these into account, I decided to investigate the association between MDM2 and S6Ks, initially *in vitro* with the use of recombinant proteins and then in cellular models.

3.2.2 Testing the interaction between MDM2 and S6Ks in GST pull-down assay

The GST pull-down assay has been used extensively to examine the interaction between potential binding partners. In order to test the interaction between MDM2 and S6 kinases in the pull-down assay, I obtained a panel of GST/MDM2 fusion constructs from Prof. Karen Vousden (CRUK, Glasgow). To purify GST alone and GST/MDM2 fusions, I carried out the expression of recombinant proteins in *E. Coli* XL-1 Blue cells and purified them using affinity purification on reduced Glutathione Sepahrose. Briefly, *E. Coli* XL-1 Blue competent cells were prepared as described in section 2.2.1.2, and transformed with 10 ng of pGEX2T or pGEX2T-MDM2 plasmids (Table 3.1; Figure 3.1). Initially, I tested the expression and the solubility of GST/MDM2 fusion proteins. To do so, a single colony from each transformation was selected on ampicillin-containing agar plates, followed by inoculation in 2 ml LB broth medium containing 100 μg/ml ampicillin as a starter culture. After overnight growth, the culture was diluted in 20 ml of LB broth medium supplemented with 100 μg/ml Ampicillin, and the suspension grown at 37°C with shaking until it reached an OD_{600nm}=0.6. Expression of recombinant proteins were induced by adding IPTG at a final

concentration of 0.4 mM, and followed by incubation for 4 hours with shaking at room temperature. After incubation, bacteria cells (1ml from each transformation) were pelleted and washed with ice-cold PBS. Cells were resuspended in 1ml ice-cold lysis buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 50 mM NaF, 50 mM NaCl, and 1% (v/v) Triton X-100 supplemented with 1 mM PMSF) and sonicated on ice under mild conditions. The aliquot (50μl) of total cell lysate was removed at this stage for SDS-PAGE analysis, then, insoluble fractions from the total cell lysate were removed by centrifugation. The resulting supernatant was used for gel electrophoresis and affinity purification on Glutathione-Sepharose 4B (Pharmacia). When the fractions from the total cell lysates and supernatants were resolved by SDS-PAGE and stained by Coomassie, it became apparent that all GST/MDM2 fusions are highly expressed, but the majority of recombinant proteins remained in insoluble fractions (Figure 3.2). To find out how much recombinant protein could be purified from 1ml of induced cultures and to examine their quality, the remaining supernatant (900 μl) was affinity purified on Glutathione Sepharose (20 μl of beads), with binding carried out for 2 hours. After extensive washing, bound proteins were eluted from the beads by boiling in Laemmli sample buffer and separated by SDS-PAGE electrophoresis. As shown in Figure 3.2B, all GST/MDM2 fusion constructs could be purified from induced cultures, but with different levels of efficiency. Notably, the quality of purified proteins differed significantly, especially at the level of degraded products. For example, GST/MDM2-127 is the most stable fusion product, while the full length GST/MDM2 is the least stable. In order to improve the quality of expressed GST/MDM2 fusions, I changed the expression conditions by lowering the temperature for induction to room temperature and reducing the time for IPTG induction to 2 hrs. In addition, I have also included a cocktail of protease inhibitors (Roche) in the lysis, washing and storage buffers. These modifications allowed us to get more soluble and less degraded GST/MDM2 proteins, which could be stored at -20C for a long time without precipitation or further degradation (data not shown). To purify large quantities of GST alone and GST/MDM2 fusion proteins, the induction of expression was carried out in 500ml of bacterial cultures and the affinity purification on

Glutathione Sepharose was performed as described in section 2.2.3.1. Purified recombinant proteins were then stored at -20°C.

The GST pull-down assay was carried out using purified proteins coupled to Glutathione Sepharose and the lyates of insect cells (Sf9, *spodoptera frugiperda* 9) infected with viruses driving the expression of protein kinases PKB/Akt, S6K1 and S6K2, which are all conjugated at the N-terminus with the EE tag epitope. Forty-eight hours after infection, cells were washed in PBS and lysed in Lysis Buffer containing 100 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM EDTA, pH8.0, 50mM NaF, 1% Triton X-100 and the cocktail of protease inhibitors. The supernatants were then collected and protein concentrations measured. Three micrograms of GST/MDM2 recombinant proteins (GST/MDM2-162, GST/MDM2-293 and GST/MDM2-376) were pre-coupled with 20 µl of 50% Glutathione Sepharose suspension for 1 hour at 4°C. After removing non-bound proteins by washing in binding buffer, the lysates of Sf9 cells infected with viruses for PKB/Akt, S6K1 or S6K2 were added to beads and incubated on the wheel at 4°C for 2 hours. After washing 3 times with lysis buffer, the associated proteins were separated by SDS-PAGE and immunoblotted with anti-EE antibodies. The supernatants of Sf9 cells infected with PKB/Akt, S6K1 or S6K2 baculoviruses were also loaded on the gel as controls. Figure 3.3 clearly demonstrates that all three kinases were well expressed in Sf9 cells infected with corresponding baculoviruses and have the predicted molecular weights (lanes 13-15). Consistant with previous reports, PKB/Akt kinase showed specific interaction with all three GST/MDM2 fusions (lanes 1-4), but not with GST alone (Zhou *et al.*, 2001;Mayo and Donner, 2001;Ashcroft *et al.*, 2002). This experiment has also revealed a direct association between MDM2 and both S6K1 and S6K2 (lanes 7-8 for S6K1 and 11-12 for S6K2). Notably, the association between MDM2 and S6Ks under these experimental conditions is more apparent than that with PKB/Akt.

Taken together, the GST pull-down assay provided the first indication of specific interaction between MDM2 and S6K1/2. My results encouraged further

Plasmid	Insert	Express system
pcDNA-S6K1 I	Wild-type S6K1 I	Mammal
pcDNA-S6K1 II	Wild-type S6K1 II	Mammal
pcDNA-S6K2 !	Wild-type S6K2 I	Mammal
pcDNA-S6K2 II	Wild-type S6K2 II	Mammal
S6K1 ΔNΔC	Rapamycin-resistant S6K1	Mammal
S6K2 ΔNΔC	Rapamycin-resistant S5K2	Mammal
pcDNA-PKB	Wild-type PKB/Akt	Mammal
pGEX-MDM2 56	GST/MDM2 1-56	Bacteria
pGEX-MDM2 127	GST/MDM2 1-127	Bacteria
pGEX-MDM2 162	GST/MDM2 1-162	Bacteria
pGEX-MDM2 207	GST/MDM2 1-207	Bacteria
pGEX-MDM2 236	GST/MDM2 1-236	Bacteria
pGEX-MDM2 293	GST/MDM2 1-293	Bacteria
pGEX-MDM2 376	GST/MDM2 1-376	Bacteria
pGEX-MDM2 WT	GST/I 22 wild-type	Bacteria
pGEX-MDM2 166A	GST/MDM2 S166A mutation	Bacteria
pGEX-MDM2 186A	GST/MDM2 S186A mutation	Bacteria
pcDNA-MDM2 WT	Wild-type MDM2	Mammal
pcDNA-MDM2 166A	S166A mutation of MDM2	Mammal
pcDNA-MDM2 186A	S186A mutation of MDM2	Mammal
iCR1	siRNA against S6K1	Mammal
iCR2	siRNA against S6K2	Mammal
iCR6	Scrambled siRNA control	Mammal
iCR7	siRNA against PKB/Akt	Mammal

Table 3.1 List of plasmids used in the report.

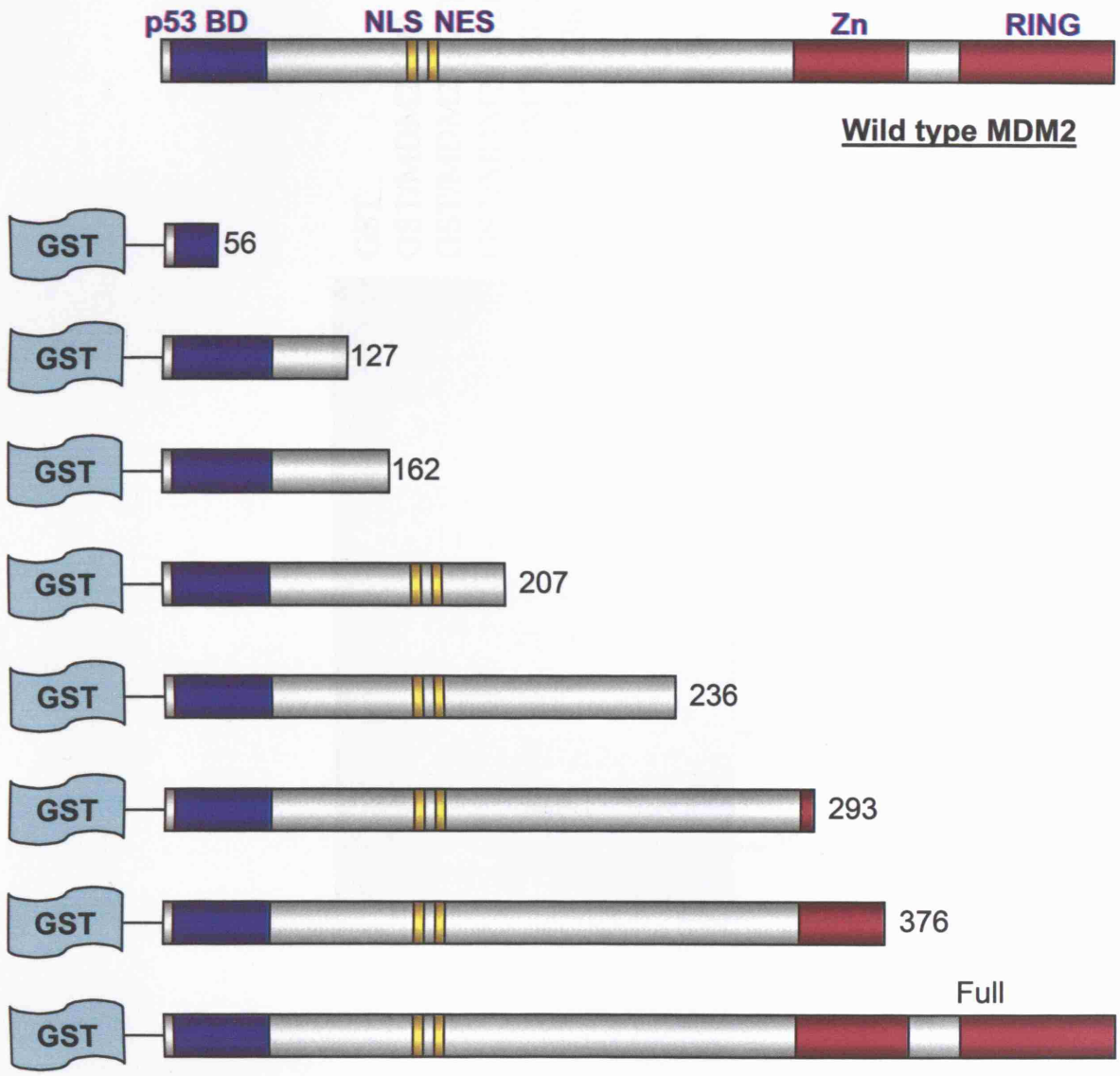


Fig. 3.1 Schematic representation of the GST/MDM2 fusion proteins used in the pull-down assay.

The p53-binding domain (p53 BD), the nuclear localization sequence (NLS), the nuclear export sequence (NES), the zinc finger (Zn), and the RING finger (RING) are indicated. The length of the MDM2 fragment from the N-terminus is indicated is at the end of each fusion construct. Note that the lines between GST and MDM2 fragments are schematic separation between the two parts of fusion proteins without indication of additional sequences.

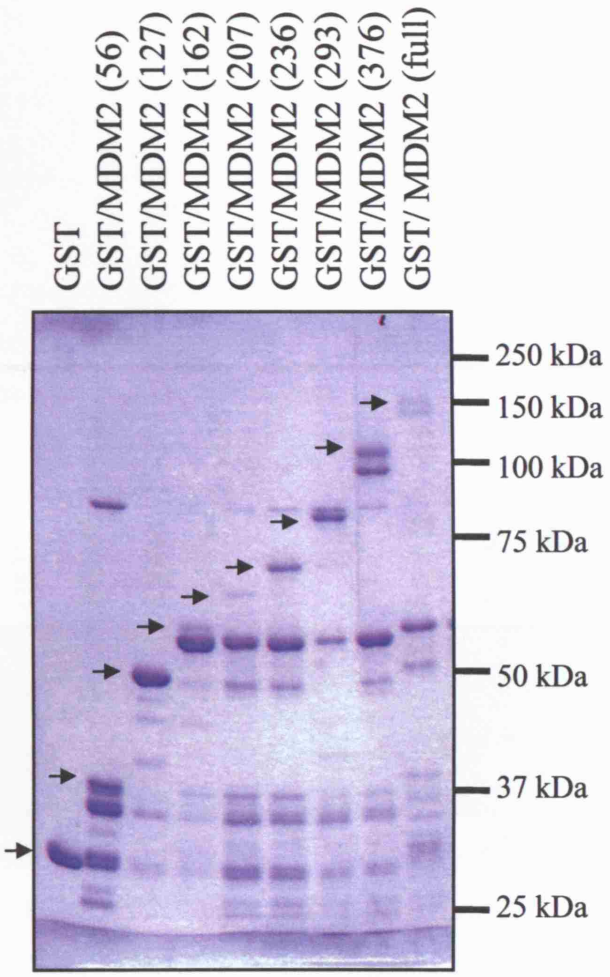


Fig. 3.2 SDS-PAGE analysis of purified GST-MDM2 recombinant proteins.

The GST-fusion MDM2 proteins were purified from *E. coli* transformed with a panel of pGEX-MDM2 plasmids. GST/MDM2 fusion proteins and GST alone were purified on Glutathione Sepharose, eluted with reduced glutathione and kept at -20C in the storage buffer as described in Section 2.2.3.1. Two micrograms of each protein was separated on SDS-PAGE and stained with Coomassie blue.

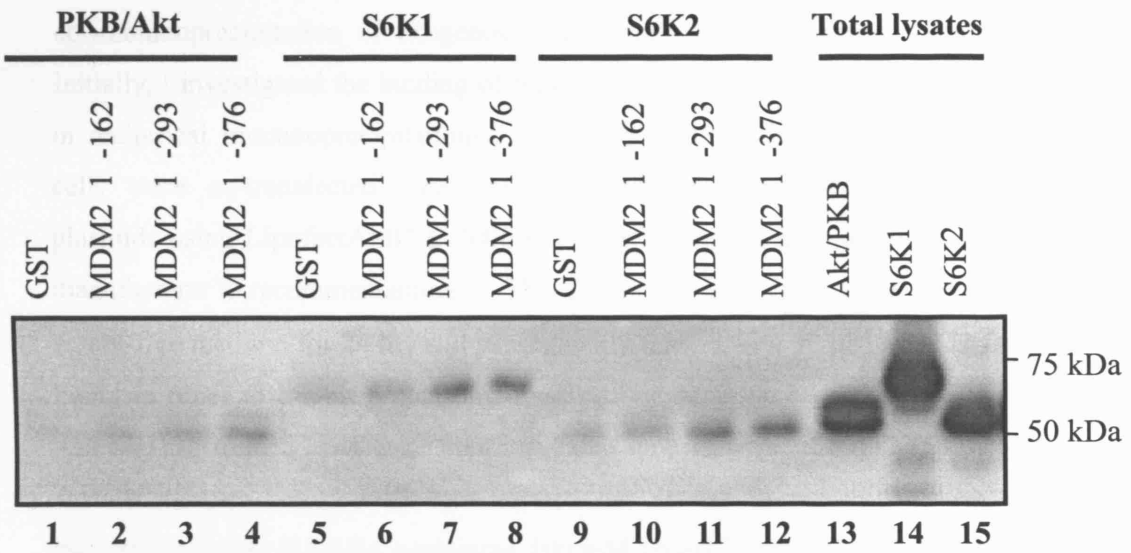


Fig. 3.3 The analysis of MDM2/S6K1/2 interaction in GST pull-down assay.

GST alone or GST/MDM2 fusions (2μg each) were coupled to Glutathione Sepharose beads. Lysates of Sf9 cells infected with recombinant viruses for EE-tagged PKB/Akt (lanes 1-4), S6K1 (lanes 5-8), and S6K2 (lanes 9-12) were incubated with the pre-coupled beads for 2 hours. After extensive washing, associated proteins were separated by SDS-PAGE and immunobloted with anti-EE antibodies. The lysates of Sf9 cells infected with EE-tagged Akt/PKB, S6K1, and S6K2 (lanes 13-15) were used as loading controls.

investigation into the specificity of binding in cell-based assays.

3.2.3 MDM2 associates with S6K in cells

The specificity of interaction between MDM2 and S6K1 *in vivo* was examined by co-immunoprecipitation of exogenously and endogenously expressed proteins. Initially, I investigated the binding of transiently expressed EE-S6K1 and MDM2 in reciprocal immunoprecipitations. To do so, exponentially growing Hek293 cells were co-transfected with pcDNA3.1/EE-S6K1 and pcDNA3.1/MDM2 plasmids using LipofectAMINE 2000 (Life Technologies, Inc.) according to the manufacturer's recommendations. The following day, cells were starved in serum-free medium for 24 hrs and subsequently treated with 10 μM MG132 for 6 hours in order to inhibit proteasome-mediated degradation of MDM2. Starved and MG132 treated cells were then incubated with or without IGF-1 (50 ng/ml) for 30 minutes. Cell lysis was carried out on ice in pre-chilled low salt association buffer (LSAB), containing 100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 % v/v Nonidet P-40 (NP-40), 1 mM sodium orthovanadate, and a cocktail of protease inhibitors (Roche). Clear cell extracts were collected following centrifugation, and immunoprecipitated with S6K1 polyclonal antibody on Protein A Sepharose beads. The immune complexes were then separated by SDS-PAGE, transferred to the PVDF membrane and probed with anti-S6K1 and anti-MDM2 antibodies. The results presented in Figure 3.4A clearly indicate that MDM2 specifically co-immunoprecipitates with S6K1 in both starved and IGF-1 stimulated cells. Notably, the level of co-immunoprecipitated MDM2 is significantly higher in cells stimulated with IGF-1, indicating that the association between S6K1 and MDM2 is inducible upon growth factor stimulation. The mobility shift of immunoprecipitated S6K1 (Figure 3.4A, lower panel) in IGF-1 stimulated cells is typical for activated S6 kinase and results from multiple phosphorylations.

In addition, I used U2OS cells which stably overexpress wild-type MDM2 (kindly provided by Prof. K. Vousden). This cell line has been used extensively to investigate *in vivo* interactions between MDM2 and its binding partners, including p53, p300, PKB/Akt. Initially, I analyzed the expression level of S6K1 in U2OS cells and found it similar to that in MCF-7 breast cancer cell line, which is known to be a high expresser of both S6K1 and S6K2 (data not shown). In this experiment, U2OS cells were starved in serum-free medium for 24hrs, treated with 10 μM MG132, a proteasome inhibitor, for a further 6 hours and then incubated with or without 10% FCS for 30 minutes. After lysing in the LSAB, the concentration of cell extracts were estimated with Coomassie Protein Reagent (Pierce) as described in section 2.2.3.3. Two hundred μg of each total lysates were used in the immunoprecipitation with S6K1 antibody, followed by Western blotting with anti-MDM2 antibody. As shown in Figure 3.4B, S6K1 immune complexes contain noticeable amounts of MDM2 in both serum-starved and serum-stimulated cells. Interestingly, the inducible nature of serum-induced association of MDM2 with S6K1 is not immediately obvious, as it was observed in HEK293 cells stimulated with IGF-1. One explanation for this result could be a significantly higher level of activated forms of S6K1 (Figure 3.4B, lower panel) in serum-starved U2OS cells, suggesting that overexpression of MDM2 may positively affect the activity of S6Ks.

To confirm the interaction between endogenous S6K1 and MDM2, I employed MCF7 cells which express both proteins at a high level. To obtain significant inhibition of S6K activity, MCF7 cells were starved for 36 hours before treatment with MG132 (10 μM for 6 hours) and subsequent stimulation with IGF1 (50 ng/ml for 30 minutes). After harvesting in LSAB buffer, cell extracts were immunoprecipitated with anti-MDM2 antibody and probed in a Western blotting experiment, using affinity purified S6K1 antibody. The results presented in Figure 3.4C clearly show that equal quantities of MDM2 were immunoprecipitated from serum-starved and IGF-1 stimulated cells. Moreover, both isoforms of S6K1 (p70, S6K1 II and p85 S6K1-I) are readily observed in IGF-1 stimulated cells. S6K1 isoforms are also present in immunoprecipitates

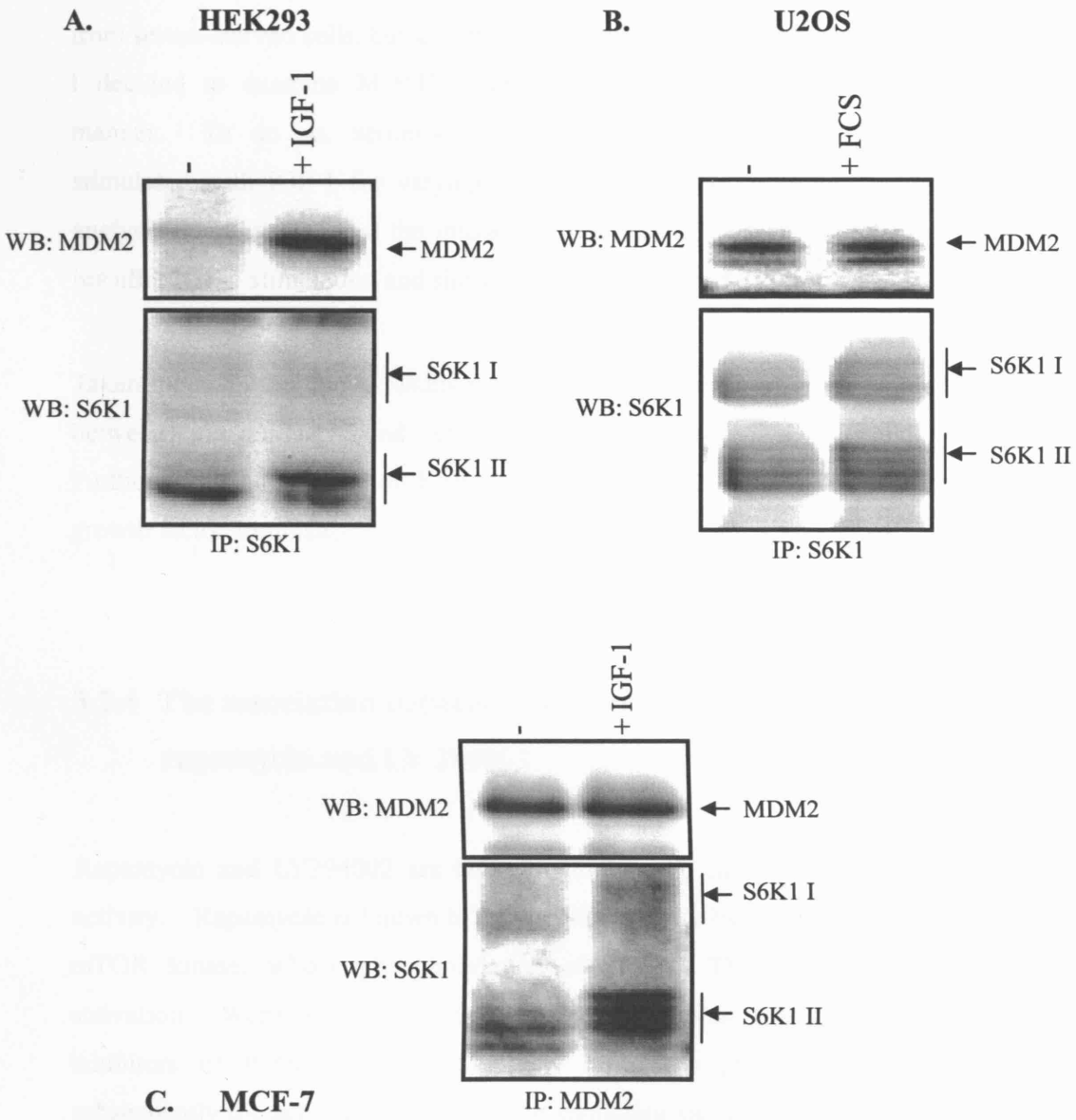


Fig 3.4 MDM2 interacts with S6K *in vivo*.

A. HEK293 cells were co-transfected with MDM2 and S6K1, starved in serum-free medium for 24 hours and treated with MG132 (10 μM) for 6 hours. After IGF-1 (50ng/ml) stimulation for 30 minutes, cell extracts were prepared with LSAB and estimated with Coomassie Protein Reagent. Two hundred μg of each lysate was immunoprecipitated with S6K1 antibody, followed by Western blotting with antibodies to MDM2 and S6K1. **B**. U2OS cells, which stably overexpress MDM2, were treated in the same way as described above for HEK293 cells, with the exception of being stimulated with 10% FCS. The S6K1 immune complexes were resolved by SDS-PAGE and probed with MDM2 and S6K1 antibodies. **C.** Exponentially growing MCF-7 cells were starved in phenol red-free DMEM medium without serum for 36 hours and treated with MG132 (10 μM) for 6 hours prior to stimulation with IGF-1 (50 ng/ml) for 30 minutes. After lysing in LSAB buffer, endogenous MDM2 was immunoprecipitated from 200 μg of each lysate and the obtained immune complexes were probed with antibodies to S6K1 and MDM2.

from serum-starved cells, but at a much lower level. Based on the data obtained, I decided to examine MDM2/S6K1 association in a time-course dependent manner. To do so, serum-starved and MG132-treated MCF7 cells were stimulated with IGF-1 for varying time periods. The co-immunoprecipitation analysis demonstrated that the interaction between MDM2 and S6K1 peaks at 2 hrs after IGF-1 stimulation and shows an inducible pattern (Figure 3.5).

Taken together, the above results provide strong evidence of specific interaction between exogenously and endogenously expressed MDM2 and S6K1. Furthermore, the association between both proteins is inducible in response to growth factor stimulation.

3.2.4 The association between MDM2 and S6Ks is affected by rapamycin and LY 294002.

Rapamycin and LY294002 are two commonly used indirect inhibitors of S6K activity. Rapamycin is known to inhibit S6K activity by blocking the activity of mTOR kinase, whose phosphorylation of S6K at Thr389 is crucial for its activation. Wortmannin and LY 294002, on the other hand, are well known inhibitors of PI3Ks. They effectively block the production of PIP_3 and subsequently the activation of two major signalling kinases, PKB/Akt and PDK1. The latter kinase phosphorylates S6K1 in the activation loop site Thr229, which culminates in a multi-step phosphorylation/activation process of S6 kinase. Since the association between MDM2 and S6K1 was found to be induced in response to IGF-1, I investigated whether the inhibition of S6K1 activity would affect the association with MDM2. In this analysis, I used HEK293 cells transiently transfected with MDM2 and S6K1. The day following transfection, cells were starved for 24 hours and incubated with 10 μM MG132 for 6 hours. LY 294002 and rapamycin were added 30 minutes before IGF-1 stimulation. Immunoprecipitation and Western blotting were carried out as described in

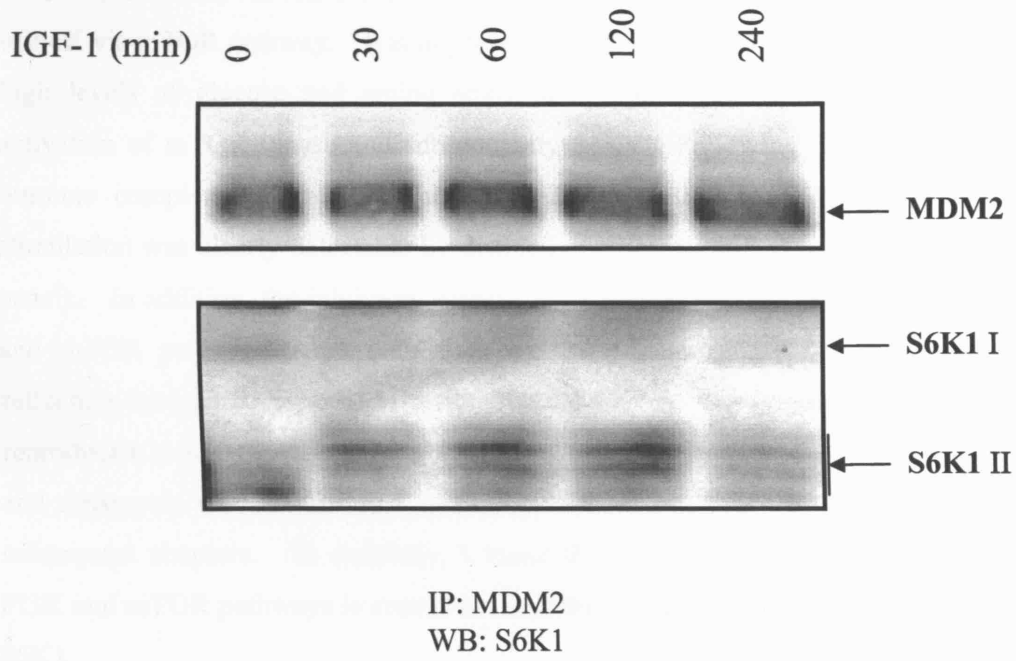


Fig. 3.5 Time-course association between S6K1 and MDM2 in MCF-7 cells.

MCF-7 cells were starved in phenol red-free DMEM medium without serum for 48 hours, followed by MG132 (10 μM) treatment for 6 hours. Cells were stimulated with IGF-1 (50 ng/ml) for the time indicated. Cellular proteins were extracted with LSAB lysis buffer and the immune complexes precipitated with MDM2 antibody. The Western blot was probed with anti-S6K1 and anti-MDM2 antibodies.

previous experiments. As shown in Figure 3.6, MDM2 specifically co-immunoprecipitates with S6K1 and this association is significantly increased by IGF-1 stimulation. However, treatment of cells with LY294002 reduces the association almost to that observed in serum-starved cells. Notably, the MDM2/S6K1 interaction is barely detectable in rapamycin treated cells. These results indicate that the background interaction between MDM2 and S6K1, observed in serum-starved cells, could be mediated by nutrient-driven activation of S6K via mTOR pathway. It is important to note that DMEM medium contains high levels of glucose and amino acids, which have been implicated in the activation of mTOR kinase and subsequently S6Ks. Following probing of the immune complexes with S6K1 antibody, the activation of S6K1 by IGF-1 stimulation was clearly detectable by distinctive mobility shift (Figure 3.6, lower panel). In addition, the inhibitory effects of LY294002 and rapamycin on PI3K and mTOR pathways were confirmed by the lack of S6K1 mobility shift, reflecting the activation status of the kinase (lanes 3 and 4). These results were reproduced in 4 independent experiments. The inhibitory effect of LY294002 and rapamycin on MDM2/S6K1 interaction will be presented in more detail in subsequent chapters. To conclude, I found that the activation signalling from PI3K and mTOR pathways is crucial to drive the interaction between MDM2 and S6K1.

3.2.5 S6K activity is present in complex with MDM2.

By confirming the interaction between MDM2 and S6K1 proteins under various experimental conditions, I was interested to find out whether or not S6K1 was also detectable in MDM2 immunoprecipitates. The S6K *in vitro* kinase assay is well established in our laboratory; therefore it was used in this project to examine the presence of S6K activity in complex with endogenous MDM2 immunoprecipitated from MCF-7. Briefly, exponential phase MCF-7 cells were starved in phenol red-free DMEM medium for 40 hours and 10μM MG132 for an additional 6 hours. Then, 50 nM rapamycin, 20 μM LY294002 or DMSO alone

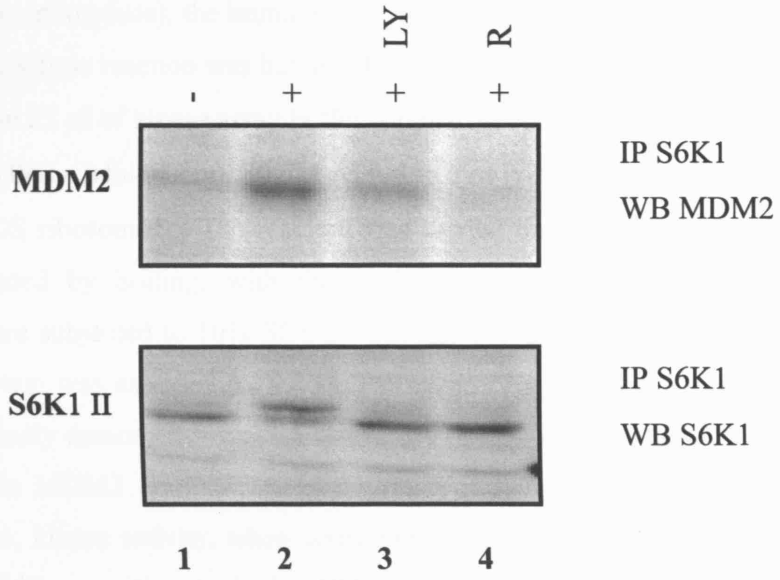


Fig. 3.6 The association between MDM2 and S6K1 is inhibited by LY294002 and rapamycin.

HEK293 cells were co-transfected with MDM2 and S6K1. One day later transfected cells were serum starved for 24 hours and treated with 10 μM MG132 for 6 hours. Then, 20 μM LY294002, 50 nM rapamycin or DMSO alone were added to cells for 30 minutes prior to IGF-1 stimulation (50 ng/ml for 30 minutes). The immunoprecipitation assay and Western blotting were carried out as described in previous experiments.

were added for 30 minutes, followed by IGF-1 stimulation (50 ng/ml, 30 minutes). Cells were harvested in LSAB lysis buffer and endogenous MDM2 protein was immunoprecipitated with anti-MDM2 antibody immobilized on protein G-Sepharose. After three washes with lysis buffer and one wash with kinase assay buffer (50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiotreitol (DTT), 10 mM β-glycerophosphate), the immune complexes were subjected to the S6 kinase assay. The kinase reaction was initiated by resuspending the beads with immune complexes in 25 μl of kinase assay buffer supplemented with 1 μM protein kinase A inhibitor (PKI, Calbiochem), 50 μM ATP, 5 μCi of [γ-^{32}P]ATP (Amersham), and 20 μg of 80S ribosomes. The reaction was carried out at 30°C for 30 minutes and terminated by boiling, with the addition of SDS-PAGE sample buffer. Samples were subjected to 10% SDS-PAGE, and the amount of ^{32}P incorporated into S6 protein was assessed by autoradiography. The results shown in Figure 3.7 undoubtedly demonstrate that S6 kinase activity towards ribosomal protein S6 is present in MDM2 immunoprecipitates from IGF-1 stimulated MCF-7 cells. Furthermore, kinase activity, when complexed with MDM2 in IGF-1 stimulated cells, is highly sensitive to both LY29400 and rapamycin. This result was repeated at least three times with MCF-7 cells, and also further confirmed in HEK293 cells (data not shown). Each of them has presented similar outcome.

In summary, I found that exogenously expressed and native S6K1 and MDM2 form specific complexes in various cell lines. The observed association occurs at low levels in serum-starved cells, but is significantly induced in response growth factor stimulation. It is also obvious from the inhibitor studies that PI3K and mTOR pathways regulate the interaction between S6K and MDM2.

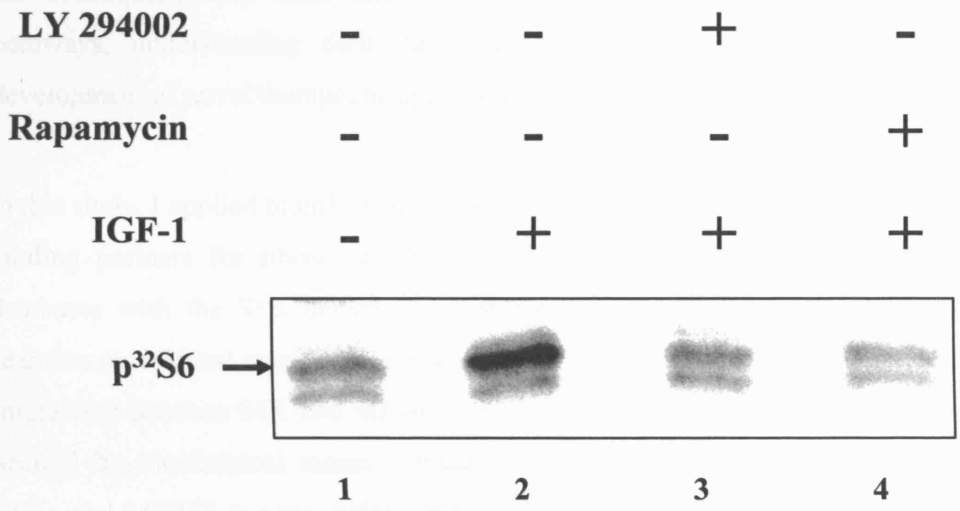


Fig. 3.7 S6K activity associates with MDM2 in MCF-7 cells.

MCF-7 cells were starved in phenol red-free DMEM medium (no added serum) for 40 hours and treated with 10 μM MG132 for 6 hours. Cells were then treated with 50 nM rapamycin, 20 μM LY294002, or DMSO for 30 minutes prior to stimulation with IGF-1 (50 ng/ml) for additional 30 minutes. Cellular proteins were extracted with LSAB lysis buffer and endogenous MDM2 was precipitated with anti-MDM2 antibody coupled with protein G Sepharose. The immune complexes were used in S6K kinase assay and the radiolabelled S6 protein was detected by radiography.

3.3 Discussion

The formation of multi-enzyme complexes, mediated by protein-protein interactions is crucial in signal transduction mediated by extracellular stimuli and in coordination of cellular responses. Knowledge of regulatory interactions and the consequences of such interactions is important for defining signalling pathways, understanding their deregulation of human pathologies and the development of novel therapeutic approaches.

In this study, I applied bioinformatic and biochemical approaches to identify novel binding partners for ribosomal S6 kinase. Bioinformatic analysis of protein databases with the S6K phosphorylation motif, followed by detailed literature searches on selected candidates, allowed me to focus on probing the specificity of interaction between S6K and MDM2 (one of the best hits from the bioinformatic search) by biochemical means. Initially, I confirmed the interaction between S6Ks and MDM2 *in vitro*, using GST pull-down assay. It is important to note that the interaction between recombinant S6K1/2 and GST/MDM2 fusions was specific, but not particularly efficient. As a positive control, I used a well-described interaction between MDM2 and PKB/Akt which belongs to the family of AGC kinases. To further validate the specificity of S6K/MDM2 binding, I employed reciprocal co-immunoprecipitation of exogenously expressed and native proteins. These studies were carried out in several cell lines and under different experimental conditions, and provided a proof for a specific association between S6K1 and MDM2 in mammalian cells. It became also apparent that the S6K1/MDM2 interaction is induced in cellular response to extracellular stimuli, such as IGF-1. This finding was confirmed by the use of indirect S6K inhibitors, LY29400 and rapamycin, which effectively block the interaction between S6K1 and MDM2. Since the activation of S6K is driven by phosphorylation-mediated conformational changes and the relocation of the activated kinase to the nucleus, one can suggest that one or both of these events are likely to facilitate the interaction with MDM2.

Finally, I showed that MDM2-associated S6K1 is in activated state, as it readily phosphorylates ribosomal protein S6, one of the well known physiological subsrates. This data, together with the bioinformatic finding of two potential S6K phosphorylation motifs in MDM2, highlight the possibility that S6K can phosphorylate and subsequently regulate the function of MDM2.

CHAPTER FOUR:

CHARACTERIZATION OF MDM2 PHOSPHORYLATION BY S6K KINASES

CHAPTER FOUR

CHARACTERIZATION OF MDM2 PHOSPHORYLATION BY S6 KINASES

4.1 Introduction

The transduction of signalling information from extracellular stimuli to various cellular compartments requires the formation of multienzyme complexes in which the message is often transduced through posttranslational modification, such as phosphorylation, acetylation, ubiquitination, methylation etc. In the previous chapter, I showed that the interaction between S6K and MDM2 occurs *in vitro* and *in vivo*, and is induced in response to mitogenic stimuli. Taking into account that both S6K and MDM2 are enzymes, I reasoned that they can modify each others functions through phosphorylation and ubiquitination respectively.

Interestingly, nearly 20% of the amino acids in the MDM2 protein are either serine or threonine residues, and the MDM2 protein is phosphorylated at multiple sites *in vivo* (Hay and Meek, 2000). The phosphorylation of MDM2 through various signalling pathways has been extensively studied during the last decade. Two clusters of phosphorylation sites have been located at the amino-terminal (amino acids 1-193) and the central (amino acid 194-293) regions of MDM2 (Hay and Meek, 2000). The first demonstration of the complex and multisite nature of MDM2 phosphorylation was by Henning et al. (Henning *et al.*, 1997), who showed that the phosphorylation status of MDM2 is influenced by early gene expression of SV40 large T antigen. Within a few years, a number of protein kinases were shown to phosphorylate and regulate MDM2. The DNA-activated protein kinase (DNA-PK) was found to mediate MDM2 phosphorylation at several sites, including Ser17, which affects its binding affinity towards p53 (Mayo *et al.*, 1997). The ATM kinase phosphorylates MDM2 on Ser395 and

impairs the degradation and nuclear export of p53 by MDM2 (Maya *et al.*, 2001). The proto-oncogene, c-Abl, and cyclin A/CDK2 complex were also reported as upstream regulators phosphorylating MDM2 at different sites.

Bioinformatic analysis revealed in MDM2 two potential phosphorylation motifs for S6K (RXRXXS/T), located in the vicinity of nuclear localization and nuclear export sequences (Ser166 and Ser186). Notably, the downstream target of PI3-kinase, PKB/Akt kinase which shares an identical phosphorylation motif with S6Ks, has been identified as one of the upstream regulators of MDM2. Recent studies revealed that PKB/Akt phosphorylates MDM2 at Ser166 and Ser186, and in turn enhances its ability to promote p53 degradation (Zhou *et al.*, 2001;Mayo and Donner, 2001;Ashcroft *et al.*, 2002;Ogawara *et al.*, 2002;Gottlieb *et al.*, 2002;Feng *et al.*, 2004). The identified S6K/MDM2 association in cells, and the presence of potential phosphorylation motifs, prompted us to investigate whether MDM2 is a novel substrate for S6K, and if so, what the consequence of this phosphorylation might be.

In this chapter, the detailed analysis of MDM2 phosphorylation by both isoforms of S6K is presented. Initially, the *in vitro* kinase assay and recombinant proteins were used to demonstrate S6K-mediated phosphorylation of MDM2. The precise mapping of phosphorylation sites was carried out with the use of MDM2 deletion mutants and mass spectrometry. These studies revealed that Ser166 is the only site in MDM2 which is phosphorylated by S6Ks. Moreover, mutational analysis of Ser166 and Ser186 and the use of phosphospecific antibodies further confirmed Ser166 as a sole site of S6K phosphorylation *in vitro* and *in vivo*.

4.2 Results

4.2.1 The *in vitro* kinase assay reveals MDM2 as a novel substrate for S6Ks

To find whether S6K is capable of phosphorylating MDM2 *in vitro*, I employed an *in vitro* kinase assay, which is a well established technique in our laboratory. The cytoplasmic forms of S6K1 (EE-tagged S6K1) and S6K2 (EE-tagged S6K2) were purified from insect cells infected with recombinant baculoviruses by affinity chromatography as previously described (Valovka *et al.*, 2003). The quality of purified recombinant S6 kinases was tested by SDS-PAGE and in *in vitro* kinase assay with ribosomal S6 protein as a substrate. As shown in Figure 4.1, affinity purified EE-S6K1 and EE-S6K2 efficiently phosphorylate S6 protein in the 80S ribosome fraction. To investigate phosphorylation of MDM2 by S6Ks I used GST alone or GST/MDM2 fusion proteins, covering the full length protein and different regulatory domains within it (GST/MDM2 1-127; GST/MDM2 1-162; GST/MDM2 1-376 and GST/MDM2 full length). In this assay, 1 μg of each of the GST fusions was mixed with 0.5 μg of recombinant S6K1 or S6K2 in kinase buffer (50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiotreitol (DTT), 10 mM β-glycerophosphate) supplemented with 1 μM protein kinase A inhibitor, 10 μM of cold ATP, and 3 μCi of [γ-^{32}P]ATP. The reaction was carried out at 30°C for 30 minutes and terminated by boiling in 1× SDS-PAGE sample buffer. The reaction mix was subjected to 10% SDS-polyacrylamide gel electrophoresis, and the amount of ^{32}P incorporated into S6 protein was assessed by autoradiography and quantified by phosphoimaging (Bio-Rad). Subsequent analysis of the autographs clearly indicated that two GST/MDM2 fusion proteins were phosphorylated by both S6K1 and S6K2 (Figure 4.2A). These were GST-MDM2 1-376 and full-length GST-MDM2. The phosphorylation of GST-MDM2 full length is visibly weaker than that of GST-MDM2 1-376. This could be explained by a lower level of GST-MDM2 full length protein in the

kinase assay, as it is highly unstable and degrades during purification (see also Chapter 3). Notably, no phosphorylation signal was observed with GST alone, while GST-MDM2 1-127 and GST-MDM2 1-162 showed some background phosphorylation. This experiment also demonstrated that MDM2 is the better substrate for S6K2 when compared to S6K1. The Coomassie analysis of the reaction mix adds support to this finding, since there was less S6K2 than S6K1 in the kinase assay (Figure 4.2B). These findings were highly reproducible, confirmed in at least 6 independent experiments.

In summary, I showed that both S6K1 and S6K2 can efficiently phosphorylate MDM2 *in vitro*. Moreover, the site(s) of S6K-mediated phosphorylation are located in the region from amino acid residues 162 until the end of the coding sequence.

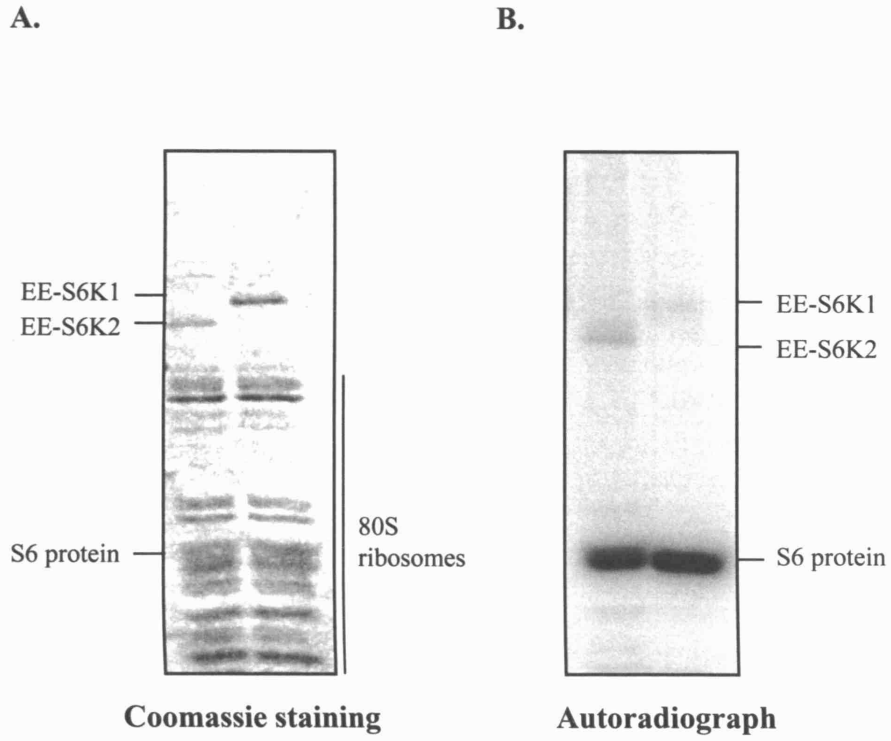


Fig. 4.1 Baculovirally expressed and affinity purified activated forms of S6K1 and S6K2 efficiently phosphorylate ribosomal protein S6 *in vitro*.

The recombinant EE-S6K1 and EE-S6K2 were expressed in insect cells infected with corresponding baculoviruses. The EE-tag monoclonal antibody coupled to Protein A Sepharose were used for affinity purification of recombinant kinases. 0.5 μg of purified EE-S6K1 and EE-S6K2 was separated by SDS-PAGE and stained by Coomassie (**A**). The activity of recombinant kinases was examined by *in vitro* kinase assay using ribosomal S6 protein as a substrate (**B**).

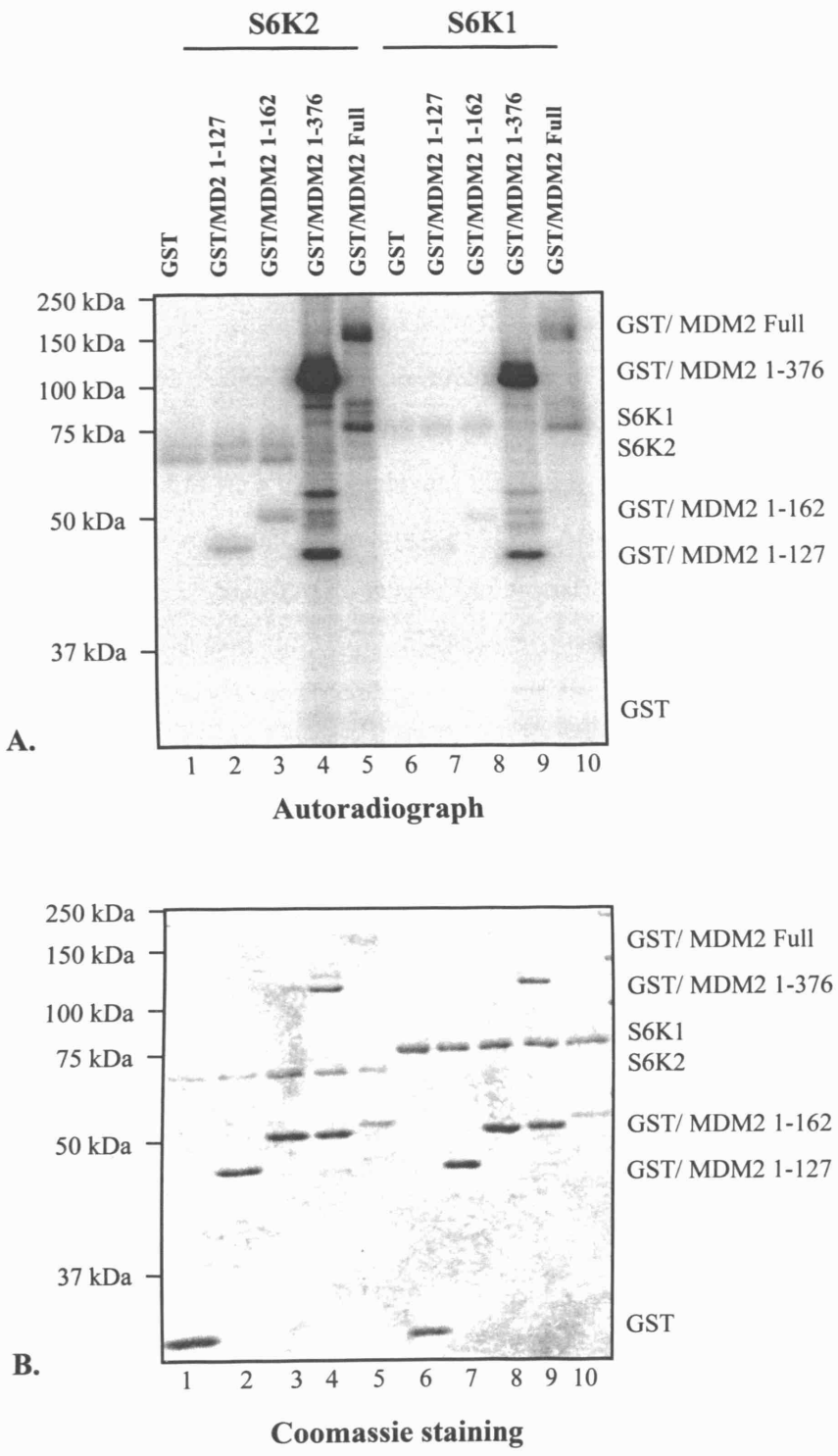


Fig. 4.2 S6K1 and S6K2 phosphorylate MDM2 *in vitro*.

The *in vitro* kinase assay was carried out in the presence of 0.5 µg of recombinant S6 kinases and 1 µg GST/MDM2 proteins as described in Material and Methods. The reaction mixtures were separated by SDS-PAGE, stained with Coomassie blue and exposed to the X-ray film. The autoradiography of S6 kinase assay (**A**) and the Coomassie staining of the proteins used in the kinase assay (**B**).

4.2.2 Identification of S6K phosphorylation site(s) in MDM2 by mass spectrometry.

To precisely map the site(s) of S6K phosphorylation in MDM2, mass spectrometry of *in vitro* phosphorylated MDM2 was performed. In this analysis, I used GST-MDM2 1-376, as both predicted sites of S6K phosphorylation are located in the covered region of this fusion protein. 2 μg of GST-MDM2 1-376 was phosphorylated *in vitro* by recombinant EE-S6K1 or EE-S6K2 (1 μg each) as described in section 4.2.1 but without [γ-^{32}P]ATP. After terminating the reaction by the addition of 5× SDS-PAGE sample buffer and heating for 5 minutes, the samples were subjected to 10% SDS-polyacrylamide gel electrophoresis. Coomassie-stained bands corresponding to GST-MDM2 1-376 were cut from the gel and used for mass spectrometry analysis. The analysis of generated samples was carried out by Heike Rebholz (member of our laboratory) using tandem MS on a quadrupole time-of-flight mass spectrometer (QStar, PE Sciex) equipped with a NanoESI source. Briefly, the tryptic digest of phosphorylated MDM2 was desalted on C18 Zip Tips (Millipore) and eluted in 1% formic acid, 50% acetonitrile and 49% water, then loaded into gold-coated nanospray needles (New Objectives, PicTip). Analysis of the resulting peptide masses clearly indicated the presence of only one doubly charged phosphopeptide of m/z 924 in samples phosphorylated by S6K1 and S6K2. The potential phosphopeptide was selected for fragmentation and the characteristic fragmentation ions of the y- and b-series were clearly detected, in addition to a peptide representing the precursor ion minus a phosphate group (HPO_3^{3-} of 80m/z) (Figure 4.3). Markedly, the peptide corresponding to the location of Ser186 was found in the analysis, but did not contain a phosphate group. The non-phosphorylated form of GST-MDM2 1-376 was used as a negative control in this analysis. No phosphopeptide was found in this sample.

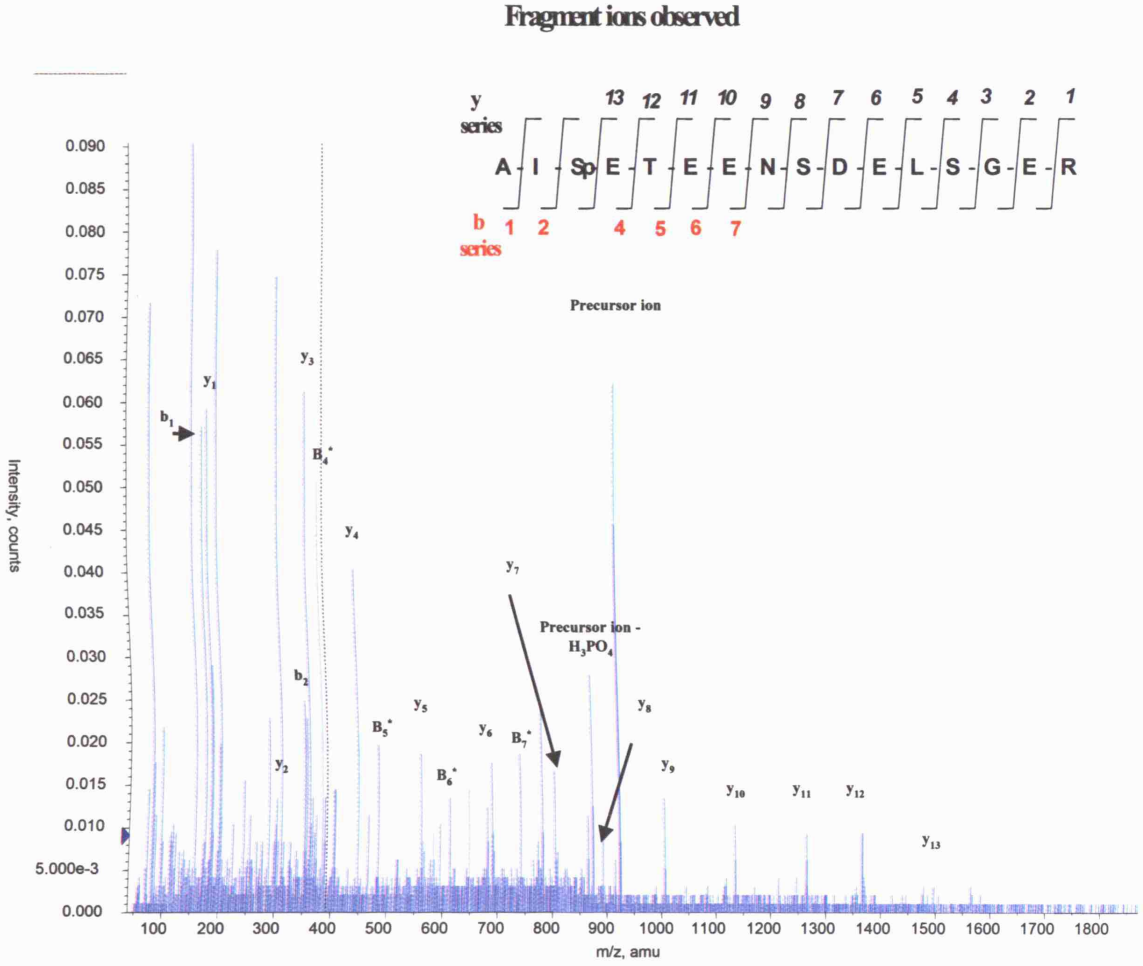


Fig. 4.3 Identification of Ser166 in MDM2 as the phosphorylation site for S6Ks by mass spectrometry.

For sequencing the phosphopeptide of a mass of 1765.77 Da, which had been identified in precursor ion scan, tandem MS on a quadrupole time-of-flight mass spectrometer (QStar, PE Sciex) equipped with a NanoESI source was performed. The tryptic digest was desalted on C18 Zip Tips (Millipore) and eluted in 1% formic acid, 50% acetonitrile and 49% water. A few microliters were loaded into gold-coated nanospray needles (New Objectives, PicTip). The doubly charged phosphopeptide of m/z 924 was selected for fragmentation and the characteristic fragmentation ions of the y- and b-series could be detetecd in addition to a peptide representing the precursor ion minus a phosphate group (HPO3 of 80 m/z).

4.2.3 Ser166, but not Ser186, is the site on which MDM2 is phosphorylated by S6 kinases

To confirm the results of mass spectrometry, indicating that Ser166 is the only site of S6K phosphorylation in MDM2, I carried out *in vitro* kinase assay with MDM2 mutants in which Ser166 and Ser186 were substituted for alanine. Taking into account that bacterially expressed full length MDM2 is not particularly stable, I carried out the purification and *in vitro* kinase assay. I included a cocktail of protease inhibitors in the lysis, washing and storage buffer, and also reduced the time of purification on Glutathione Sepharose. Following these measures, the resulting quality of purified GST-MDM2 wild type and mutants was higher (Figure 4.4 upper panel). Purified proteins were incubated with EE-S6K1 and EE-S6K2 in the presence of radioactive ATP and a standard kinase buffer. As shown in Figure 4.4 (lower panel) both S6Ks phosphorylate wild type MDM2 and S186A mutant with the same efficiency. However, the phosphorylation of S166A mutant is barely detected, indicating that S6Ks can *in vitro* phosphorylate only one site in MDM2, which is Ser166. It is important to note that these results are consistent with the mass spectrometry analysis. Markedly, MDM2 fusion constructs were found to be more suitable substrates for S6K2, when compared with S6K1. My results were reproduced in two independent experiments.

In summary, I have provided conclusive evidence that MDM2 is the substrate for S6Ks *in vitro*. S6K-mediated phosphorylation of MDM2 at Ser166 was identified by mass spectrometry and further confirmed by mutational analysis followed by *in vitro* kinase assay.

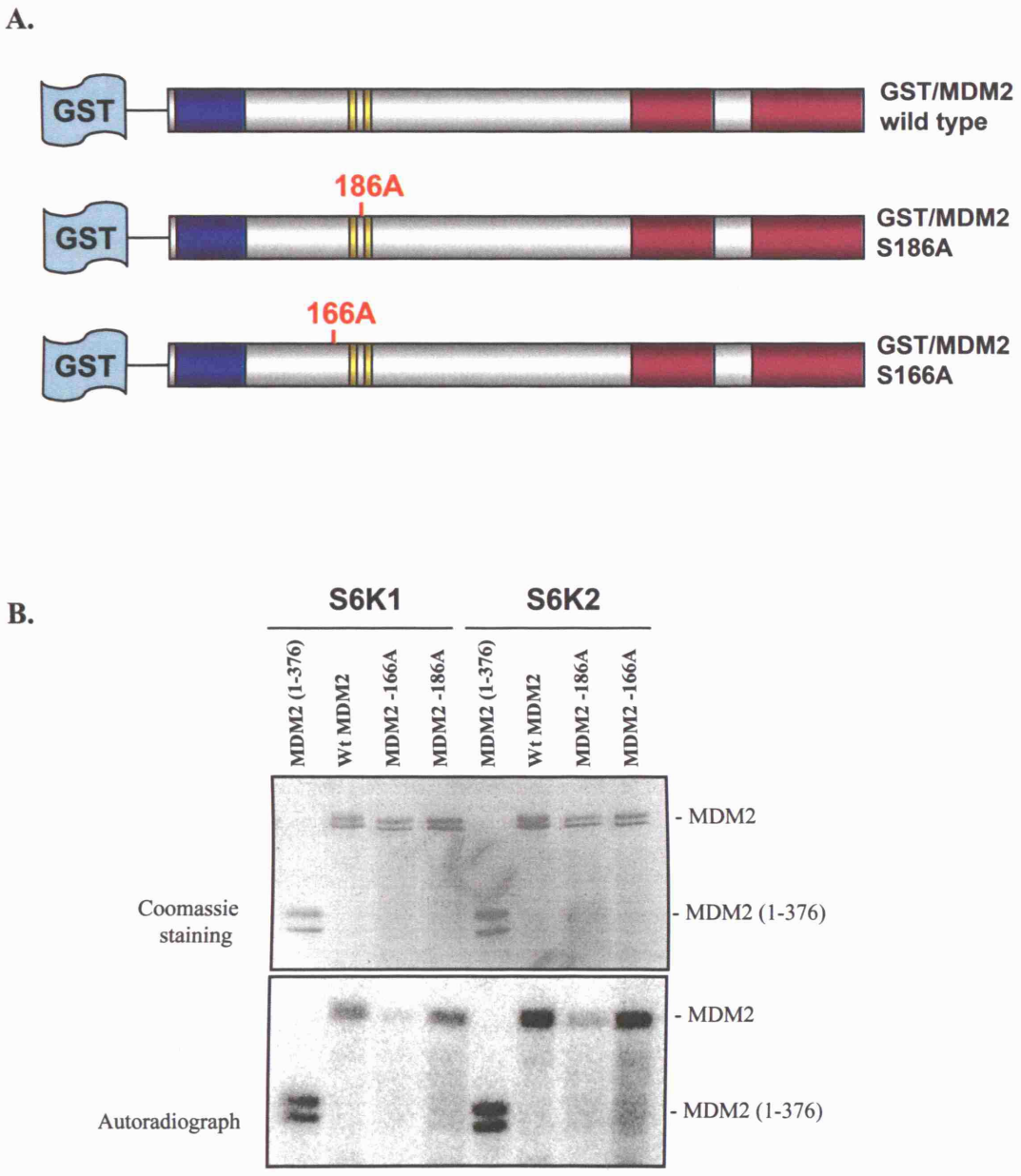


Fig. 4.4 Ser166, but not Ser186, is phosphorylated by S6Ks.

A. Schematic presentation of GST/MDM2 fusion constructs used in this analysis (GST/MDM2-WT, GST/MDM2-S166A, GST/MDM2-S186A, GST/MDM2 1-376). Note that the lines between GST and MDM2 fragments are schematic separation between the two parts of fusion proteins without indication of additional sequences. **B.** *In vitro* S6 kinase assay was carried out in the presence of 0.5 μg of GST fusion proteins as described in previous experiments. The amount of GST/MDM2 fusion proteins used in the assay is shown on Coomassie stained gel (upper panel). The autoradiography shows the level of phosphorylation of GST/MDM2 fusion proteins.

4.2.4 Generation and characterization of phosphospecific antibody directed against pS166 in MDM2.

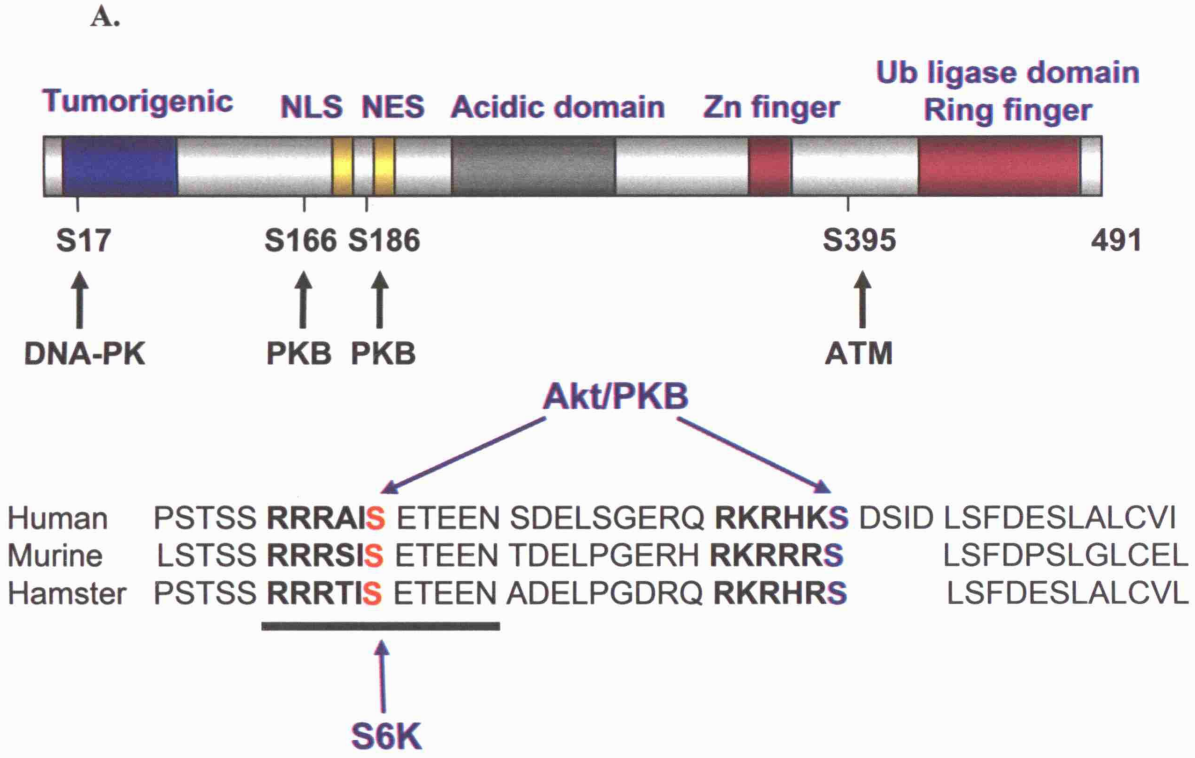
Phosphospecific antibodies have been used extensively to analyze the physiological relevance of phosphorylation/dephosphorylation events on cellular proteins in different signalling pathways. To confirm *in vivo* phosphorylation of MDM2 at Ser166 and to elucidate its importance, I attempted to generate polyclonal phosphospecific antibodies directed to the identified site of phosphorylation. The pS166-MDM2 antibody was not commercially available, so I ordered its production in Eurogentec, a company which specializes in developing monoclonal and polyclonal antibodies. Briefly, the synthetic phosphorylated peptide, corresponding to MDM2 sequence around Ser166 (CRRRAI**pS**ETEEN) was coupled to KLH and used for immunization of rabbits according to a standard protocol. The titre of antibodies was analyzed by ELISA assay using phosphorylated (CRRRAI**pS**ETEEN) and non-phosphorylated (CRRAI**S**ETEEN) peptides on the matrix. After five rounds of immunization, the rabbits were sacrificed and their sera used for affinity purification of phosphospecific antibodies.

I carried out affinity purification and characterization of pS166-MDM2 antibodies. Initially, I prepared affinity columns by coupling phosphorylated (CRRRAI**pS**ETEEN) and non-phosphorylated (CRRAI**S**ETEEN) peptides to Actigel beads. Then, generated sera were loaded onto the columns containing phosphorylated peptide. After extensive washing, specifically associated IgG were eluted from the beads in borate buffer pH 3.0. After neutralizing the pH with 1M Tris HCL pH 8.0, protein concentration was measured in eluted fractions. In order to remove IgGs which recognize non-phosphorylated forms of the peptide, eluted fractions were loaded onto the column containing CRRAI**S**ETEEN peptide. The flow-through was collected, dialysed against 1xPBS with 50% Glycerol and stored at -20C. The specificity of pS166-MDM2 antibody was analyzed by Western blotting as follows. NIH3T3 cells were transfected with

either MDM2-WT or MDM2-166A using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's recommendations. Transfected cells were then treated with 10 μM MG132 for 5 hours prior to harvest. The total cell lysates were prepared with LSAB lysis buffer and subjected to immunoblotting with affinity purified pS166-MDM2 antibody. The same membrane was subsequently reblotted with MDM2 antibody to assess the amount of total MDM2 protein. As shown in Figure 4.5B, affinity purified antibody specifically recognized MDM2-WT, but not MDM2-166A, which lacks an S6K phosphorylation site. Soon after pS166-MDM2 antibody generation, it became commercially available from Cell Signalling. Therefore, I have used both antibodies in the duration of this project, as they both demonstrated similar specificity towards *in vivo* phosphorylated MDM2 at Ser166.

4.2.5 MDM2 is phosphorylated at Ser166 in response to various extracellular stimuli

The availability of specific pS166-MDM2 antibody allowed us to examine the phosphorylation status of this site in cellular response to various extracellular stimuli and signal transduction inhibitors. In this analysis, I commonly used cell lines, HEK293 (human embryonic kidney 293) and MCF-7 (breast cancer cell line). Exponentially growing HEK293 and MCF-7 cells were serum starved for 24 hours, followed by treatment with 10 mM MG132 for 5 hours. Then, cells were incubated in the presence or absence of FCS, growth factors or mitogens (10% FCS, 50 ng/ml IGF-1, 0.1 μM insulin, 40 ng/ml PDGF, 1 μM TPA, and 50 ng/ml EGF) for 1 hour. The lysates of harvested cells were immunoblotted with a panel of antibodies, including pS166-MDM2. The results presented in Figure 4.6 clearly demonstrate that in both cell lines, MDM2 phosphorylation at Ser166 increases in response to mitogenic stimulation. This is more obvious in HEK293 cells, where the level of Ser166 phosphorylation is not detectable in the serum-starved cells, but is easily seen following stimulation with FCS, IFG-1,



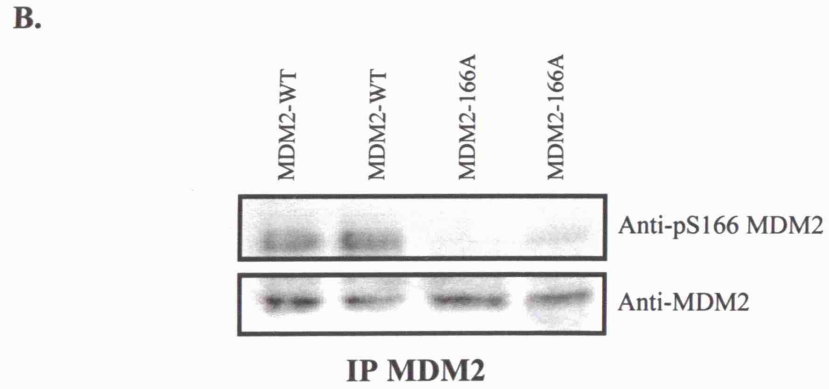


Fig. 4.5 Testing the specificity of generated pS166-MDM2 antibody.

A. Domain organization and the location of major regulatory sites of phosphorylation in MDM2. The MDM2 sequence used for making pS166 antibody is underlined. **B.** Testing the specificity of pS166-MDM2 antibody by immunoblotting. NIH3T3 cells were transfected with MDM2-WT or MDM2-166A. Cell were treated with 10 μM MG132 for 5 hours prior to harvest. The total cell lysates were prepared with LSAB lysis buffer and subjected to Western Blot analysis with pS166 MDM2 antibody. The same membrane was subsequently reblotted with MDM2 antibody.

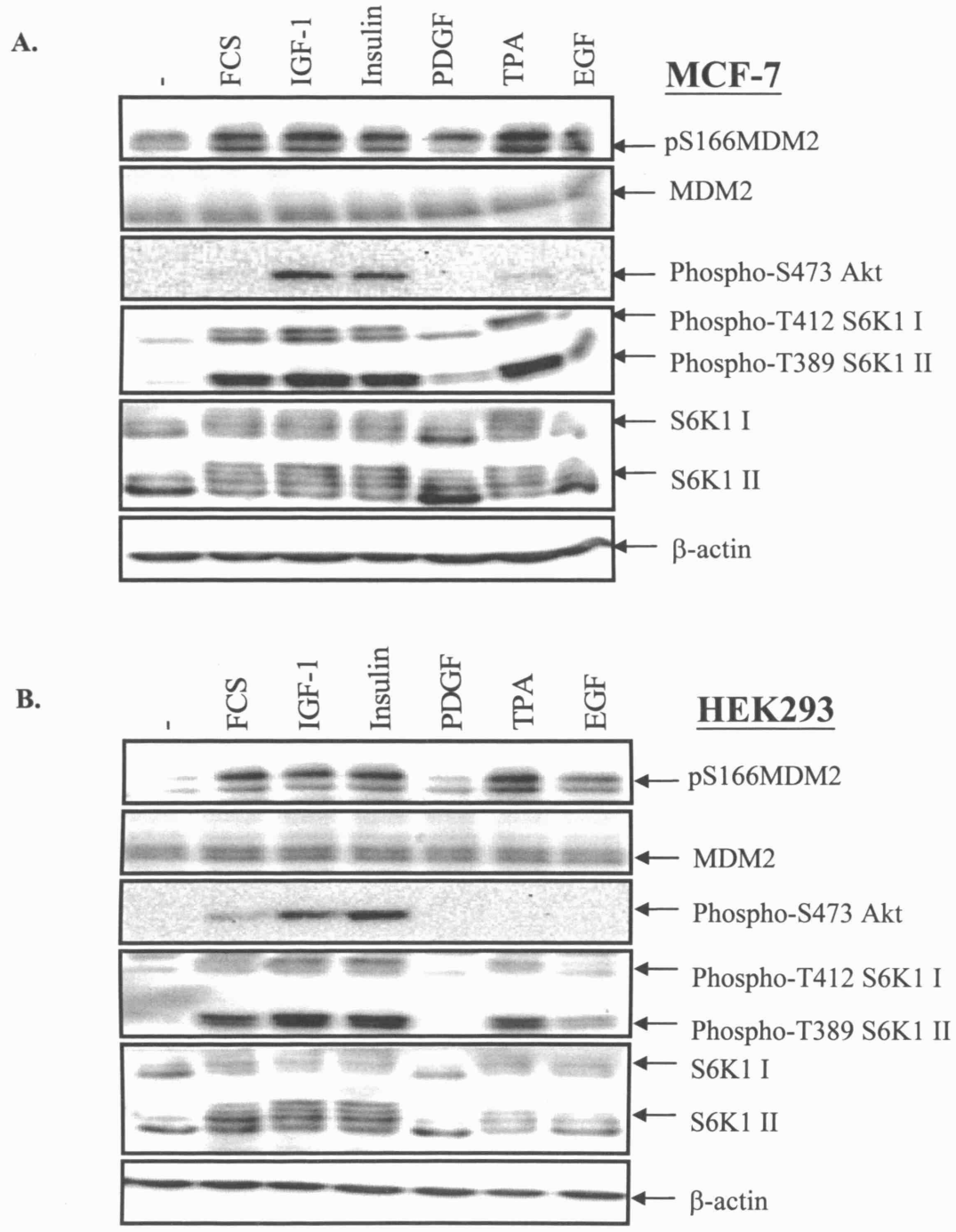


Fig. 4.6 MDM2 is phosphorylated at Ser166 in response to various mitogenic stimuli.

Serum starved MCF-7 (**A**) and HEK293 (**B**) cells were treated with 10 μM MG132 for 5 hours prior to treatment with various mitogens (10% FCS, 50 ng/ml IGF-1, 0.1 μM insulin, 40 ng/ml PDGF, 1 μM TPA, and 50 ng/ml EGF) for 1 hour. Cells were lysed in LSAB lysis buffer and total proteins (50 μg) were subjected to SDS-PAGE and Western blot analysis with a panel of antibodies.

Insulin, EGF and TPA. The background pS166 phosphorylation observed in serum-starved MCF-7 cells could be explained by strong autocrine stimulation in this cell line. When the lysates were probed with anti-MDM2 antibody, I found that the total level of MDM2 was very similar in all analyzed samples (Figure 4.6 A and B). In addition, anti-actin immunoblotting confirmed comparable protein loading volumes from each cell lysate.

Phosphospecific antibodies to PKB/Akt (pS473) and S6K (pS389/412) are often used as probes for testing the activation of signalling pathways in response to various extracellular stimuli. Probing the cell lysates with both antibodies clearly indicated that both cells lines respond to all mitogenic stimuli, with the exception of PDGF. Interestingly, the pattern of pS389/412 phosphorylation in cytoplasmic (S6K1 II) and nuclear (S6K1 I) forms of S6K1 correlated much better with pS166-MDM2 in both cell lines, when compared to pS473-PKB/Akt. This is particularly noticeable in cells stimulated with TPA and EGF. The outcome of this experiment was supported by two independent experiments.

In summary, the data presented above clearly demonstrate that generated pS166-MDM2 antibody specifically recognizes MDM2, phosphorylated at Ser166 in mammalian cells. Moreover, the phosphorylation of Ser166 is induced when serum-starved cells are treated with mitogenic stimuli. Interestingly, I observed closer correlation between the pattern of pS166-MDM2 and pS389/412-S6K1 phosphorylation than that of pS473-PKB/Akt. This was an interesting observation which prompted us to investigate it in more detail.

4.2.6 Rapamycin reveals the correlation between MDM2 phosphorylation at Ser166 and S6 kinase activation

To elucidate the contribution of PI3K/PKB and mTOR/S6K pathways in the regulation of Ser166 phosphorylation in MDM2, I employed two commonly used

inhibitors of these signalling pathways, LY294002 and rapamycin respectively. It is well established that PI3K specific inhibitor blocks the activation of PKB/Akt and S6Ks, while mTOR inhibitor rapamycin potently prevents S6K activation, but not that of PKB/Akt. Taking this into account, I treated exponentially growing PC-3 cells with DMSO, rapamycin, and LY294002 for 24 hours. Then, cells were lysed in LSAB buffer and analyzed by immunoblotting with pS166-MDM2 antibody. Remarkably, the phosphorylation of MDM2 at Ser166 was blocked by rapamycin much more efficiently than by PI3K inhibitor LY294002. As shown in Figure 4.7, 10 nM rapamycin completely halts MDM2 phosphorylation at Ser166. This result closely correlates with the inhibition of S6K activity, as observed by a faster mobility shift in the presence of rapamycin. The mobility shift ladder of S6K1 and, to a lesser extent S6K2, corresponds well with the multisite phosphorylation/dephosphorylation of S6Ks. The results I obtained clearly indicate that the inhibition of S6K activity by rapamycin abrogates Ser166 phosphorylation, while PKB/Akt is still fully active, as shown in the immunoblot with pS473-PKB/Akt.

To further investigate this phenomenon, the dose-dependent effect of rapamycin on pS166-MDM2 was analyzed. In this analysis, HEK293 cells were serum-starved for 24 hours and incubated in the presence or absence of various concentrations of rapamycin (1 nM, 10 nM, 50 nM, and 200 nM) or DMSO for an additional 5 hours. Then, cells were stimulated with 10% FCS for 1 hour and lysed in LSAB buffer. When total cell lysates were analyzed by Western blotting with pS166-MDM2 antibody, the phosphorylation of MDM2 at Ser166 was clearly induced by serum stimulation (Figure 4.8). Pre-treatment of serum-starved cells with even very low doses of rapamycin (1 nM), resulted in significant inhibition of the serum-induced phosphorylation of Ser166, while doses of 10 nM or more abolished it completely. In addition, I found that phosphorylation of S6K at Ser389 and its downstream substrate S6 protein fully correlates with the pattern of Ser166 phosphorylation in MDM2. In contrast, the pattern of PKB/Akt phosphorylation at Ser473 exhibited an opposite correlation to that of Ser166 in MDM2. The increase in rapamycin concentration stimulated

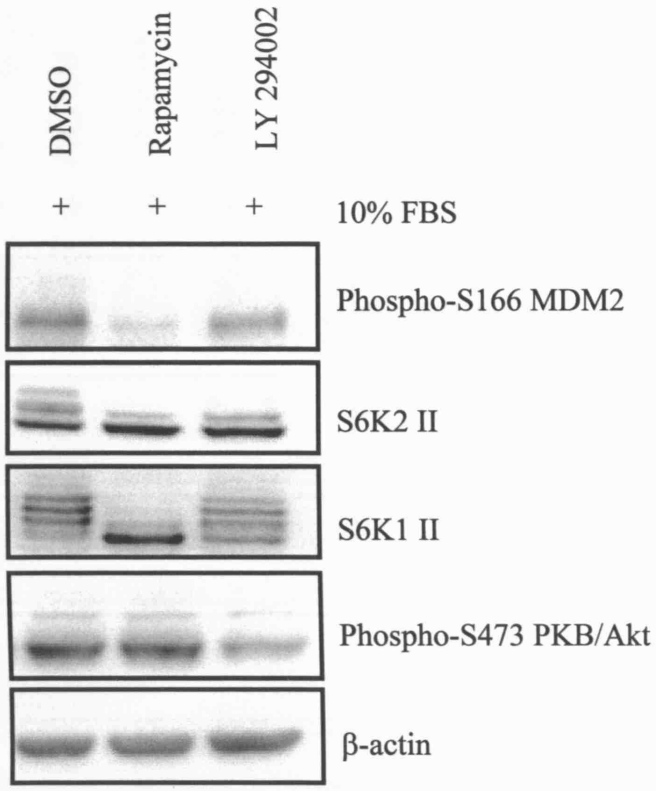


Fig. 4.7 Rapamycin is much more potent inhibitor of MDM2 phosphorylation at Ser166 than LY294002.

Exponentially growing PC-3 cells were cultured in 60 mm dishes with RPMI medium supplemented with 10% FBS. Rapamycin (10 nM), LY294002 (5 μM) or DMSO were incubated with cells for 24 hours. Total cellular lysates were analysed by Western blot analysis using indicated antibodies. Probing with anti-actin antibody was used as a loading control.

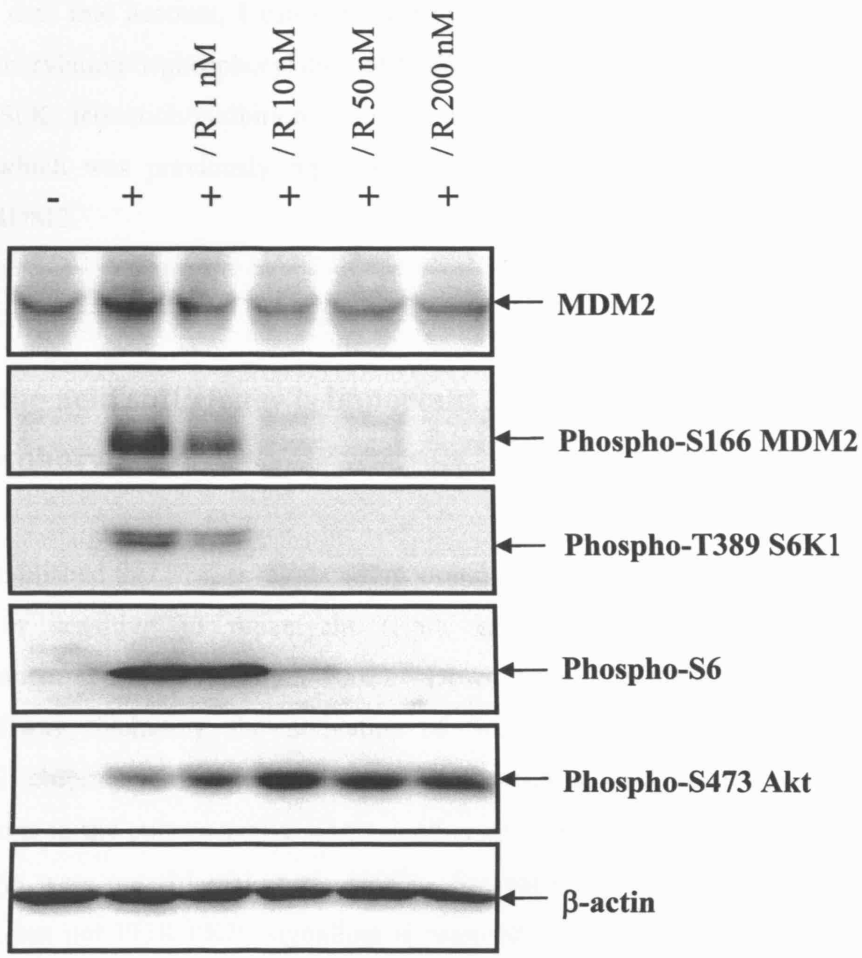


Fig. 4.8 Dose-dependent effect of rapamycin on MDM2 phosphorylation at Ser166.

HEK293 cells were cultured in the absence of serum for 24 hours and then incubated in the absence or presence of different concentration of rapamycin (R) for 5 hours. Following treatment with or without serum for 1 hour, total proteins were extracted with LSAB lysis buffer and subjected to Western blot analysis with specific antibodies.

PKB/Akt phosphorylation at Ser473, leading to its activation.

Taking this data into account, I can conclude that, in at least two different cell lines, phosphorylation/dephosphorylation of MDM2 at Ser166 closely follows the pattern of S6K activation/inhibition. So far, this has not been the case for PKB/Akt, which was previously reported to phosphorylate both Ser166 and Ser186 in MDM2.

4.2.7 Amino acid sufficiency is important for MDM2 Ser166 phosphorylation

It is well established that S6K is regulated by amino acid availability and that this regulation is sensitive to rapamycin (Hara *et al.*, 1998;Iiboshi *et al.*, 1999;Shigemitsu *et al.*, 1999;Proud, 2002). Downstream signalling events via mTOR pathway, including the activation of S6K and phosphorylation of translational inhibitor 4E-BP1, were shown to be dependent on the concentration of amino acids in the culture media, whereas other growth-related protein kinases and pathways were not (Iiboshi *et al.*, 1999). Several reports have also shown that mTOR, but not PI3K/PKB, signalling is required for amino acid-dependent activation of S6K. For that reason, amino acid deprivation serves as an experimental technique that can distinguish TOR signalling to S6K from that mediated by PI3K/PKB pathway.

Taking this into account, I were keen to explore whether or not amino acid induced signalling can affect MDM2 phosphorylation at Ser166. Two cell lines in particular, MCF-7 and HEK293, have often been used in analogous studies. In this case, I used MCF-7 cells, maintained in DMEM medium supplemented with 10% FBS. Upon reaching 70% confluence, cells were starved in phenol red-free medium without serum for 24 hours. To remove amino acids, cells were incubated in D-PBS supplemented with 20 μM MG132 for 2 hours. Essential

amino acids, with or without rapamycin (10 nM), were added back to the cells and incubated for an additional 30 minutes. Total protein extracts were prepared with LSAB lysis buffer, separated by SDS-PAGE and immunoblotted with a panel of antibodies (anti-MDM2, anti-pS166 MDM2, anti-S6K1, anti-pT389 S6K1, anti-pS6 protein, and anti-β-actin).

The outcome of this experiment is shown in Figure 4.9 and was also reproduced in HEK293 cells (data not shown). As expected, the addition of essential amino acids to nutrient-deprived cells resulted in the activation of S6K1 phosphorylation at Ser389 and strong phosphorylation of its physiological downstream substrate ribosomal protein S6. Moreover, the increase in S6K1 and S6 phosphorylation was sensitive to rapamycin. When the lysates were probed with pS166-MDM2 antibody, the amino acid-induced increase in MDM2 phosphorylation at Ser166 was evident and reproducible. Rapamycin sensitivity of amino acid-induced phosphorylation at Ser166 further confirmed my original observation that mTOR/S6K pathway regulates phosphorylation status of MDM2 at Ser166.

4.2.8 Rapamycin-resistant forms of S6Ks partially rescue the effect of rapamycin on Ser166 phosphorylation

The activation of S6K is dependent on mTOR pathway and therefore is sensitive to mTOR specific inhibitor rapamycin. So far the structure of mTOR kinase has not been solved, and the mechanism of rapamycin-driven inhibition of the kinase activity and downstream signalling is not known. In the absence of this knowledge, mutational and biochemical studies revealed critical amino acids/regions responsible for the rapamycin inhibitory affect on mTOR, and its downstream substrates. Based on these findings, rapamycin-resistant forms of mTOR and S6 kinases have been generated. As discussed in the introduction, the substitution of Ser2035 with any amino acid larger than Ala blocks binding of FKBP12-rapamycin complex to the FRB domain and therefore renders mTOR

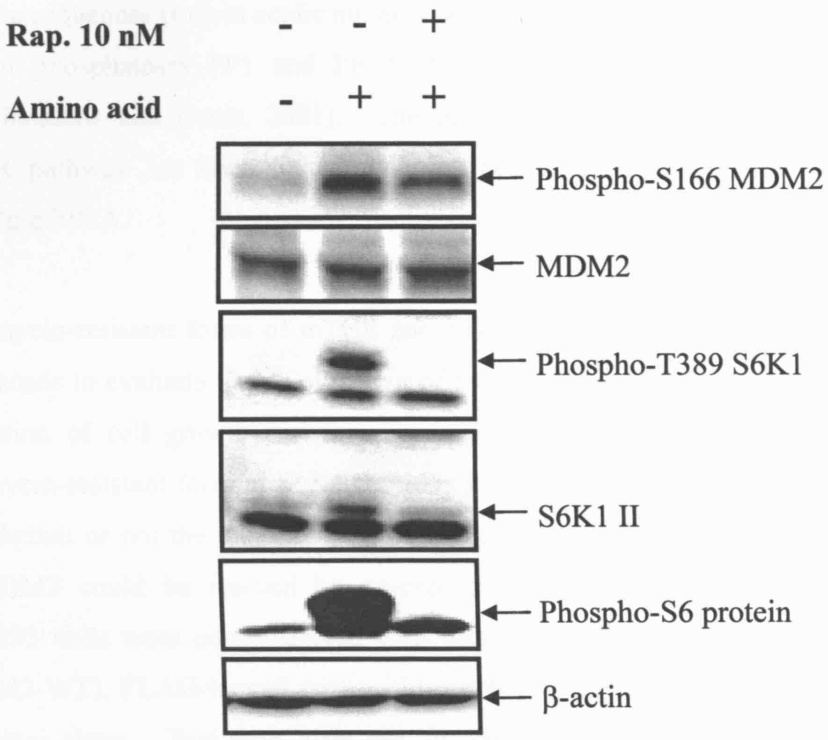


Fig. 4.9 Phosphorylation of MDM2 at Ser166 is induced by amino acids.

MCF-7 cells were seeded in 60 mm plates and cultured until reaching 70% confluency. Cells were starved in phenol red-free DMEM medium without serum for 24 hours. To remove the amino acids, cells were incubated in D-PBS supplemented with 0.1 g/L $CaCl_2$ and 20 μM MG132 for additional 2 hours. Cells were stimulated by adding essential amino acids in the absence or presence of rapamycin (10 nM) for 30 minutes. Total protein were extracted in LSAB lysis buffer and subjected to Western blot analysis with specific antibodies.

resistant to rapamycin. In the case of S6K, it was found that the deletion of both the amino- and carboxyl-terminal regulatory regions makes S6Ks insensitive to rapamycin (Figure 4.10A). It is believed that the N-terminal region possesses specific sequences (rich in acidic amino acids), which mediate the interaction with protein phosphatases PP1 and PP2A (Peterson *et al.*, 1999;Westphal *et al.*, 1999;Janssens and Goris, 2001). The inhibitory effect of rapamycin on the mTOR pathway has been linked with the activation of protein phosphatases, including PP2A.

Rapamycin-resistant forms of mTOR and S6K have been used extensively in the last decade to evaluate the involvement of their binding partners/substrates in the regulation of cell growth and the progression of the cell cycle. I obtained a rapamycin-resistant form of S6K1 from Dr. K. Yonezawa with the aim of finding out whether or not the inhibitory effect of rapamycin on Ser166 phosphorylation in MDM2 could be rescued by co-expression of this construct. To do so, HEK293 cells were co-transfected with plasmids coding for wild type MDM2 (MDM2-WT), FLAG-tagged rapamycin-resistant form of S6K1 (Flag-RR-S6K1) or vector alone. Two days after transfection, cells were serum-starved for 24 hours and incubated in the absence or presence of rapamycin (150 nM) for an additional 60 minutes. Finally, cells were stimulated with or without 10% FCS for 1 hour and harvested in LSAB lysis buffer. After clearing the lysates by centrifugation, 50 μg of each lysate was loaded on 7.5% SDS-PAGE and subjected to Western blot analysis with specific antibodies (anti-MDM2, anti-pS166 MDM2, anti-FLAG tag, anti-pT389 S6K1, and anti-β-actin) (Figure 4.10 B). The amount of pS166-MDM2 was quantified by densitometry, standardized with β-actin and shown as a relative value to the pS166-MDM2 signal in serum-stimulated cells without rapamycin treatment under each condition (Figure 4.10 C). The induction of Ser166 phosphorylation by serum stimulation and the inhibitory effect of rapamycin were confirmed once again in cells co-transfected with MDM2 and vector alone. However, when both MDM2 and Flag-RR-S6K1 were expressed in cells, the inhibition of MDM2 phosphorylation at Ser166 was significantly reduced. Approximately 50%

rescue was reproducibly achieved in four independent experiments. It is important to note that the rescue of Ser166 phosphorylation was more pronounced, when the dose of rapamycin was reduced to 50 nM. The expression of Flag-RR-S6K1 and its phosphorylation at Thr389 was confirmed by immunoblotting with anti-Flag and anti-pS389-S6K1 antibodies. Immunoblotting with anti-β-actin antibody served as a protein-loading control.

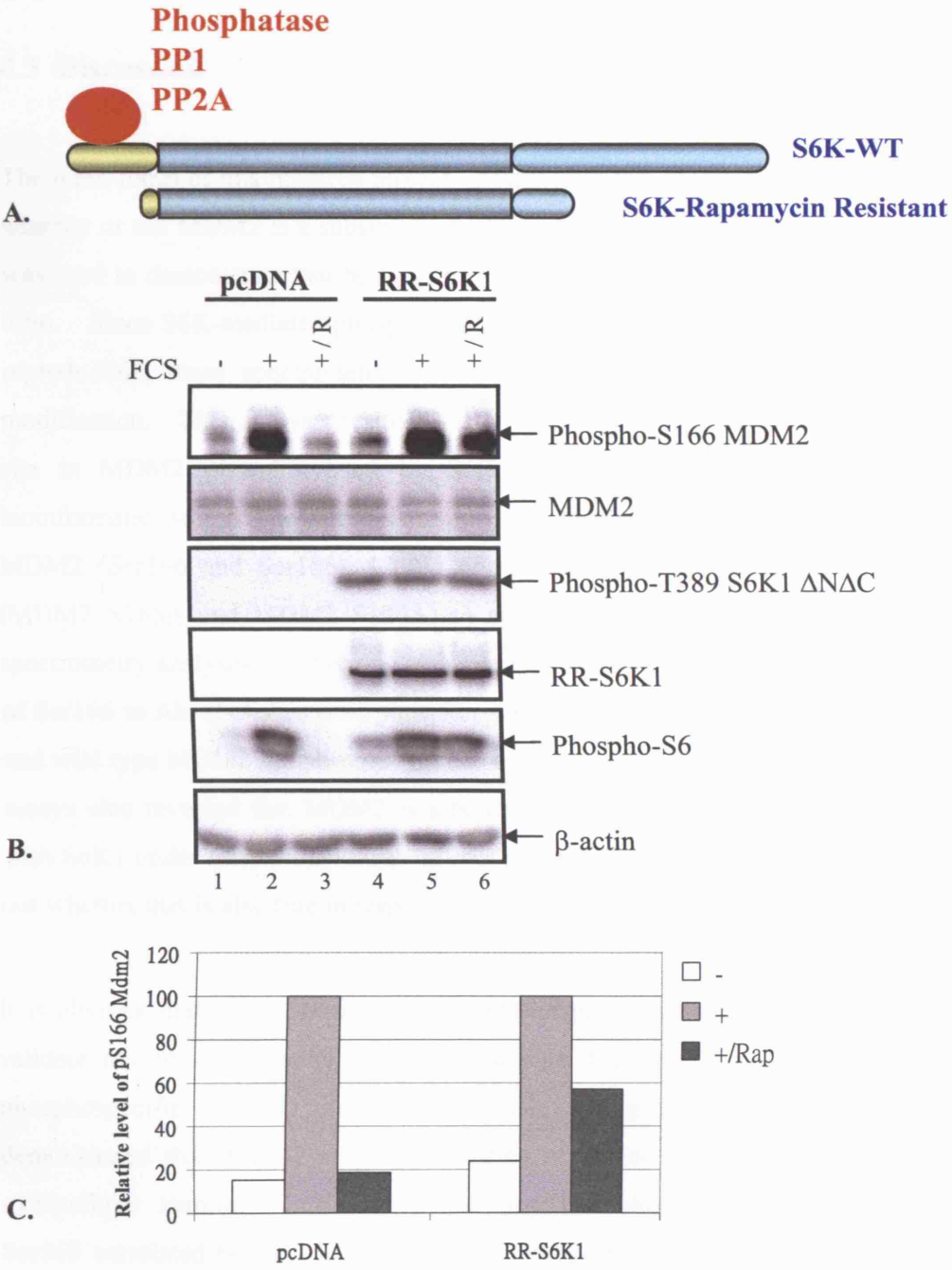


Fig 4.10 Rapamycin-resistant form of S6K1 rescues the effect of rapamycin on MDM2 phosphorylation at Ser166.

A. Schematic presentation of wild type and rapamycin-resistant forms of S6K1. **B.** MDM2 phosphorylation at Ser166 in the presence of rapamycin is rescued by rapamycin-resistant form of S6K1. HEK293 cells were transiently transfected with wild type MDM2 either together with empty vector (pcDNA) or plasmid encoding rapamycin-resistant form of S6K1 (RR-S6K1). After serum starvation, cells were incubated in the absence or presence of rapamycin (150 nM) for 1 hour and then stimulated with 10% FCS. Cell lysates were subjected to immunoblotting with a panel of antibodies. **C.** The amount of pS166-MDM2 was quantified by densitometry, standardized with β-actin and shown as a relative value to the p166-MDM2 signal in serum-stimulated cells without rapamycin treatment under each condition.

4.3 Discussion

The main focus of this research project, presented in this chapter, was to find out whether or not MDM2 is a substrate for S6K. Initially, the *in vitro* kinase assay was used to demonstrate that both isoforms of S6K can phosphorylate MDM2 *in vitro*. Since S6K-mediated phosphorylation of MDM2 was efficient and highly reproducible, mass spectrometry was employed to pin down the site(s) of modification. This analysis resulted in the identification of Ser166 as the only site in MDM2 phosphorylated by S6K1 and S6K2 *in vitro*. Since the bioinformatic search uncovered two potential S6K phosphorylation motifs in MDM2 (Ser166 and Ser186), I used non-phosphorylating mutants of MDM2 (MDM2 S166A and MDM2 S186A) to confirm the data obtained from mass spectrometry analyses. It became apparent from this experiment that substitution of Ser166 to Ala abolishes S6K phosphorylation of MDM2, while S186A mutant and wild type MDM2 are phosphorylated to the same extent. The *in vitro* kinase assays also revealed that MDM2 is a better substrate for S6K2 when compared with S6K1 under similar experimental conditions. It would be interesting to find out whether this is also true *in vivo*.

It is obvious that not all *in vitro* identified phosphorylations occur *in vivo*. To validate my *in vitro* results in cellular models, I generated and characterized phosphospecific antibody directed to Ser166. Using this antibody, I initially demonstrated that MDM2 is phosphorylated at Ser166 in response to various extracellular stimuli. Intriguingly, the observed phosphorylation pattern at Ser166 correlated better with that of Ser389/412-S6K1, than Ser473-PKB/Akt, which are commonly used as the readouts of S6K1 and PKB/Akt activation respectively. For examples, TPA and EGF clearly enhanced phosphorylation of S6K1 at Thr389/412 and MDM2 at Ser166, while the induction of Ser473 phosphorylation in PKB/Akt was hardly detectable.

PKB/Akt-mediated phosphorylation of MDM2 *in vitro* and *in vivo* was recently

reported in several independent studies (Zhou *et al.*, 2001;Mayo and Donner, 2001;Ashcroft *et al.*, 2002;Ogawara *et al.*, 2002;Gottlieb *et al.*, 2002;Shu *et al.*, 2007;Vassilev, 2007). However, some inconsistencies remain with regards to phosphorylation sites and their physiological relevance. Some studies describe phosphorylation of both Ser166 and Ser186 by PKB/Akt, while others claim that Ser186, but not Ser166 is the site for PKB/Akt mediated signalling to MDM2 (Ogawara *et al.*, 2002). In addition to these sites, phosphorylation of Ser188 has been also shown to be mediated by PKB/Akt (Feng *et al.*, 2004;Milne *et al.*, 2004). In this chapter, I provide several lines of evidence indicating that *in vivo* phosphorylation of MDM2 at Ser166 is controlled by the mTOR/S6K pathway and is directly executed by S6Ks. First of all, I showed that Ser166 phosphorylation is inhibited by rapamycin in a dose-dependent manner. Secondly, phosphorylation of MDM2 at Ser166 is strongly enhanced by amino acids, which involve mTOR/S6K pathway, but not PI3K/PKB. Thirdly, rapamycin-resistant form of S6K1 partially rescues the inhibitory effect of rapamycin on serum-induced Ser166 phosphorylation. Finally, I found in a number of studies and under various experimental conditions that the pattern of MDM2 phosphorylation at Ser166 correlates more closely with that of S6K1, than PKB/Akt. In some cases, I observed an opposite correlation for PKB/Akt (dose-course of rapamycin treatment).

In summary, the findings presented in Chapters 3 and 4 have led us to question the physiological relevance of mTOR/S6K signalling to MDM2, and the cross-talk with PI3K/PKB pathway. This will be addressed in the next chapter.

CHAPTER FIVE:

ELUCIDATING THE PHYSIOLOGICAL IMPORTANCE OF mTOR/S6K SIGNALLING TO MDM2

CHAPTER FIVE

ELUCIDATING THE PHYSIOLOGICAL IMPORTANCE OF mTOR/S6K SIGNALLING TO MDM2

5.1 Introduction

Mdm2 is a ubiquitin ligase whose main physiologically relevant substrate is tumor suppressor p53. It is well established that MDM2 can regulate p53 signalling primarily by: a) blocking p53 transactivation capacity through direct interaction with its transactivation domain; or b) by inducing p53 degradation through direct ubiquitination and targetting to proteasomes. Since p53 is a key player in the regulation of the cell cycle and cell survival in response to various stresses, protein levels and the activity of MDM2 is kept under very tight control. The expression of MDM2 was found to be enhanced by p53 at the transcription level, while PI3K/PKB signalling pathways were implicated in regulating cellular content through post-translational modifications, mainly phosphorylation. Furthermore, post-translational modifications have been also implicated in regulating MDM2 E3 ubiquitin ligase activity, its subcellular localization, and the ability to interact with a number of cellular proteins intimately involved in growth regulation, cell cycle progression, and apoptosis. For example, phosphorylation of MDM2 at Ser17 by DNA-PK was reported to have a significant impact on its ability to regulate the p53 response(Mayo *et al.*, 1997). Since Ser17 is located in close vicinity to the amino-terminal p53-interacting domain of MDM2, it has been proposed that phosphorylation on this site may structurally affect the affinity of MDM2-p53 association. Another member of the PIKK family, ATM kinase was shown to phosphorylate MDM2 at Ser395 in response to cellular stresses, inhibiting its ability to mediate p53 turnover and nuclear export (Khosravi *et al.*, 1999;de Toledo *et al.*, 2000;Maya *et al.*, 2001). Furthermore, signalling via the

PI3K/PKB pathway has a profound effect on MDM2 stability, E3 ligase activity, and the association with p53 and acetyltransferase p300 (Zhou *et al.*, 2001;Ashcroft *et al.*, 2002;Feng *et al.*, 2004).

Following the discovery of S6K-mediated phosphorylation of MDM2 *in vitro* and *in vivo*, elucidating the physiological importance of identified interactions or phosphorylation became the focus of further investigation. This objective was tackled by employing various experimental approaches, such as immunofluorescence microscopy, reporter assays, siRNA knock down of protein expression, transient and stable overexpression, measuring cell proliferation and protein stability etc. In this chapter, I provide evidence that S6K-driven phosphorylation of MDM2 at Ser166 significantly increases its stability in serum stimulated cells. Furthermore, I also found that mTOR/S6K signalling regulates the expression of p21 in a p53-independent manner.

5.2 Results

5.2.1 The pools of Ser166 phosphorylated and total MDM2 are predominantly localized in the nucleus of serum-starved or serum-stimulated cells

It has been previously demonstrated that the phosphorylation of MDM2 modulates its subcellular localization. Phosphorylation of MDM2, as mediated by the PI3K/PKB pathway in response to growth factors and mitogenic stimuli, was shown to promote MDM2 relocalization from the cytoplasm to the nucleus, thus driving p53 ubiquitination and degradation (Zhou *et al.*, 2001;Mayo and Donner, 2001;Ashcroft *et al.*, 2002). These findings provided an additional mechanism by which the PI3K/PKB pathway might mediate anti-apoptotic signalling. However, the involvement of Ser166 in MDM2 nucleocytoplasmic shuttling has been recently challenged (Ogawara *et al.*, 2002). The results presented in this study show that MDM2 phosphorylation at Ser166 does not contribute to the MDM2 translocation from the cytoplasm to the nucleus in response to mitogenic stimuli.

To find out whether or not phosphorylation of MDM2 at Ser166 can affect its subcellular localization, I employed confocal immunofluorescence microscopy. In this experiment, exponentially growing cells were plated onto poly-L-lysine coated coverslips and cultured overnight. The following day, cells were transfected with pcDNA3.1/MDM2-WT and cultured in complete media for 24 hours. After starvation for a further 24 hours, cells were stimulated with 10% FCS for 1 hour, and fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Non-specific binding was blocked by incubating with 0.5 % bovine serum albumin in PBS. Primary anti-pS166 and anti-MDM2 antibodies (at various dilutions, from 1:100 to 1:10 000) were incubated with fixed cells overnight. After extensive washing with PBS, the samples were incubated for 45

minutes with goat fluorescent isothiocyanate-conjugated (FITC) anti-mouse or anti-rabbit antibodies respectively. Fluorescently labeled cells were viewed with a Zeiss LSM510 confocal microscope, and the images analyzed using the LSM510 image browser software. I consistently observed that, in serum-starved or serum-stimulated cells, transiently overexpressed wild type MDM2 is predominantly localized in the nucleus (Figure 5.1). Notably, the intensity of the MDM2 specific signal was significantly higher in serum-treated cells in comparison to that in starved cells. This could be explained by the increased stability of MDM2 in cells stimulated with growth factors and mitogenic stimuli. When cells were probed with anti-pS166 antibody, the localization of MDM2 phosphorylated at Ser166 was also mainly nuclear and showed a very similar pattern of staining to that observed for the total pool of MDM2. In addition, I also used NIH3T3 cells in the same experimental set up, but could not detect significant nucleocytoplasmic shuttling of total or Ser166 phosphorylated MDM2 in starved or serum-stimulated cells (data not shown).

5.2.2 MDM2 protein level is regulated via both PI3K and mTOR pathways

The function of cellular proteins is regulated at various levels, including gene expression (transcription, splicing, and translation), posttranslational modifications (phosphorylation, acetylation, glycosylation) and degradation (proteosomal- or lysosomal-mediated). MDM2 is an example of such regulation as: a) signalling via p53 controls MDM2 expression at the level of transcription; b) serum starvation and cellular stresses significantly decrease MDM2 half-life; and c) mitogenic stimulation is known to increase its stability. Direct phosphorylation of MDM2 by PKB/Akt at Ser166 and other sites was shown to be responsible for increasing MDM2 half-life. The mitogen-induced and PI3K-mediated accumulation of MDM2 reduces p53 protein level via ubiquitination/proteasomal degradation and inhibits the apoptosis pathway (Zhou

et al., 2001;Ogawara *et al.*, 2002;Feng *et al.*, 2004). My original findings on MDM2/S6K association/phosphorylation prompted us to investigate whether or not the mTOR/S6K signalling pathway can also stabilize MDM2 protein. Initially, I compared the effect of LY294002 (PI3K inhibitor) and rapamycin (mTOR inhibitor) on the serum-induced increase of stably overexpressed MDM2 in U2OS cells. In this experiment, exponentially growing U2OS cells (a gift from Prof. K. Vousden) were serum-starved and treated with or without rapamycin (10 nM) or LY294002 (5 μM) for 5 hours. After stimulation with 10%FCS for 1 hour, cells were lysed and total protein extracts separated by SDS-PAGE. Immunoblot analysis with anti-MDM2 antibody clearly indicated that stimulation of starved cells with serum strongly increased the level of total MDM2 (Figure 5.2A). As expected, pre-treatment of starved cells with LY294002 blocked serum-induced accumulation of MDM2 to a significant degree. Interestingly, the effect of rapamycin on the level of MDM2 protein was similar to that of LY294002. Re-probing the same membrane with anti-actin antibodies showed that an equal amount of proteins was loaded on the gel from each sample. Taking these results into account, I focused on testing the effect of rapamycin on endogenously expressed MDM2. To do so, I used MCF-7 and HEK293 cells as they express high level of endogenous MDM2. Both cell lines were grown to about 70% confluency, followed by serum starvation and the addition of DMSO, rapamycin (10 nM) or LY294002 (5 μM). Five hours later, cells were stimulated with 10% FCS for 1 hour and lysed in LSAB buffer. Total protein extracts were analyzed by immunoblotting with antibodies specific for MDM2, S6K, and β-actin. The results presented in Figure 5.2B (upper panel) clearly demonstrate that pre-treatment of cells with both LY294002 and rapamycin, but not with DMSO, significantly reduces the accumulation of MDM2 protein in response to serum stimulation. Re-probing the membrane with anti-S6K1 antibody allowed us to validate the inhibitory effect of LY294002 and rapamycin on PI3K and mTOR signalling pathways respectively (Figure 5.2B, middle panel). Furthermore, probing with anti-β-actin antibody served as the loading control (Figure 5.2B, bottom panel). These findings were confirmed in at least three independent experiments.

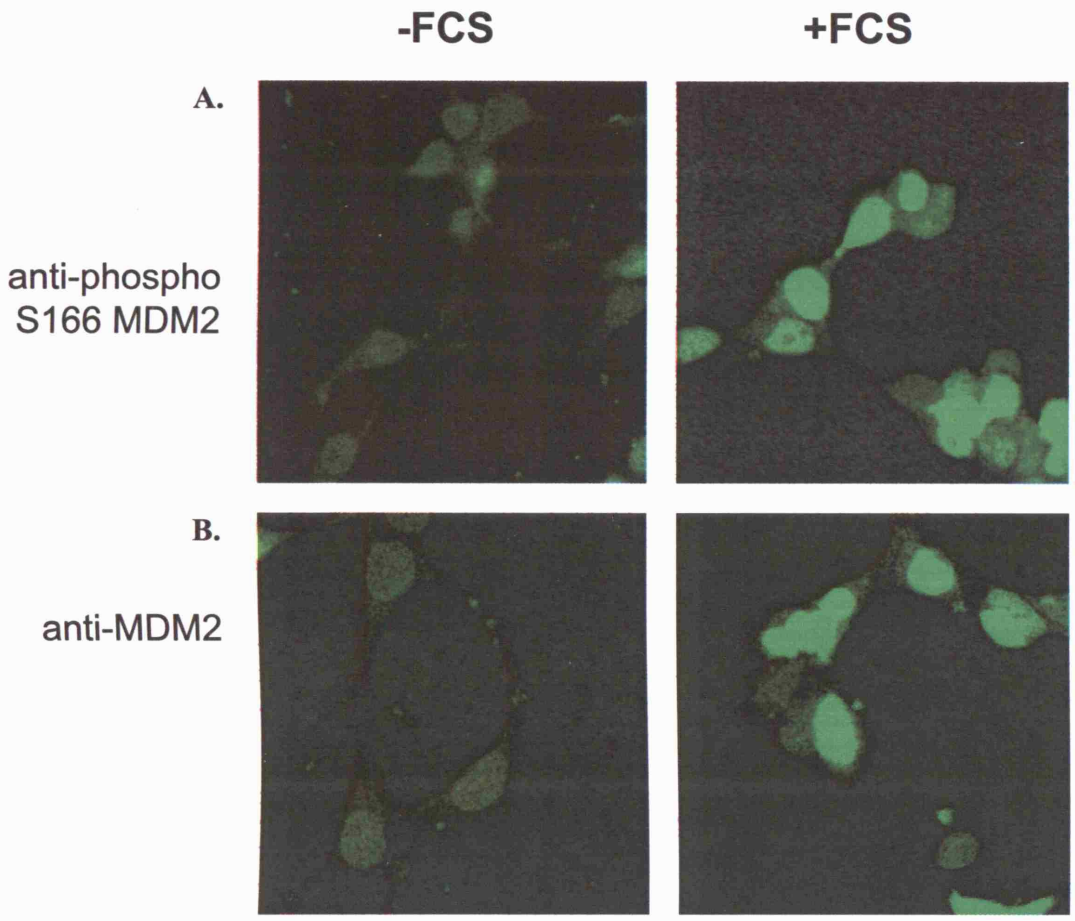


Fig. 5.1 Subcellular localization of total and Ser166 phosphorylated MDM2 does not change significantly in response to starvation or serum-stimulation.

HEK293 cells, grown on 13 mm coverslips, were transfected with pcDNA 3.1/MDM2 using ExGene500 as recommended (MBI Fermentas). Transfected cells were cultured for 24 hours and then starved without serum for an additional 24 hours. After stimulation with 10% FCS for 1 hour cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Primary anti-pS166 (**A**) and anti-MDM2 (**B**) antibodies (1:1000 dilution) were incubated with fixed cells overnight followed by probing with secondary FITC-conjugated antibody. Fluorescently labelled cells were viewed with a Zeiss LSM510 confocal microscope and the images analysed using the LSM510 image browser software.

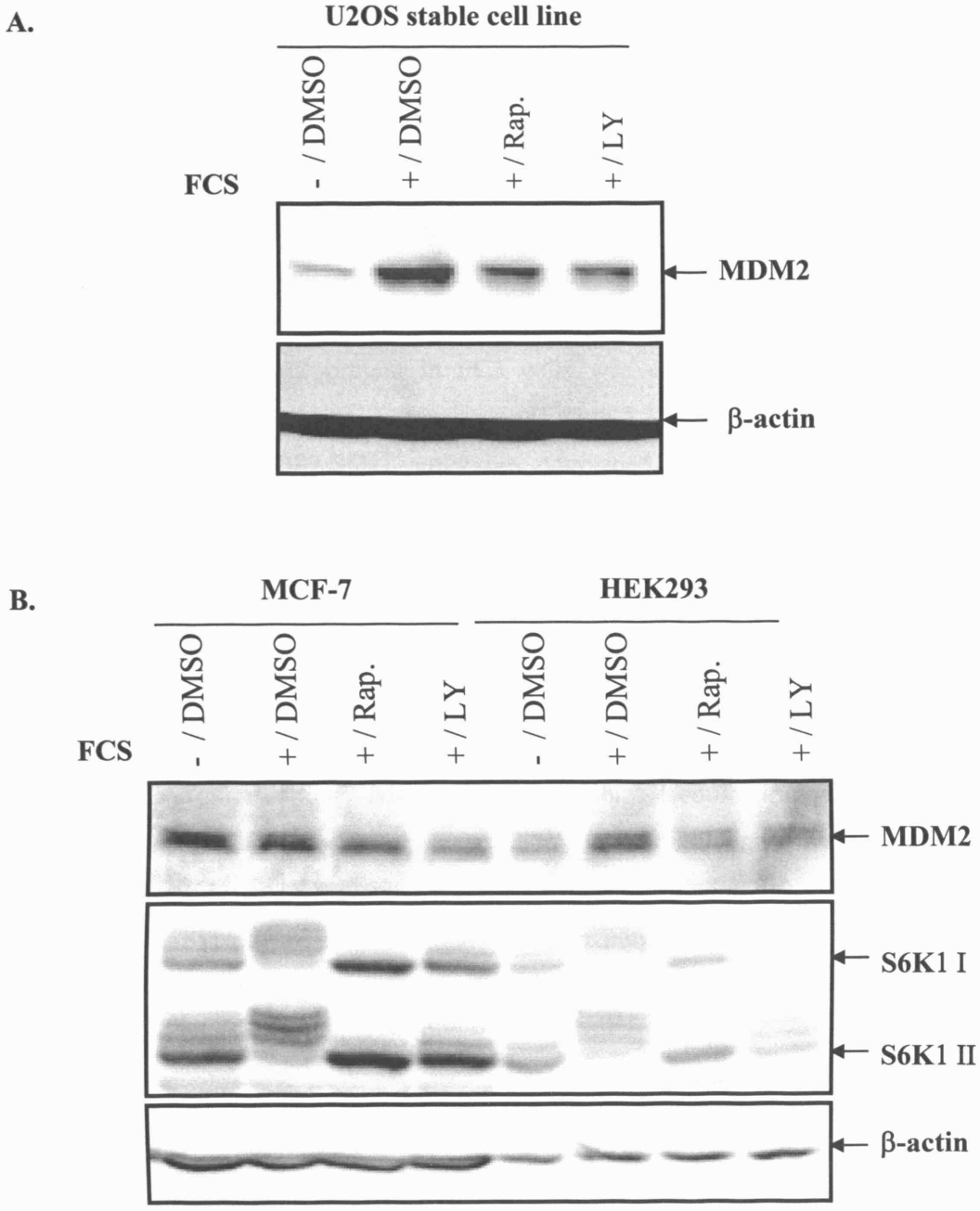


Fig. 5.2 Serum-induced accumulation of MDM2 is strongly inhibited by both LY294002 and rapamycin.

MDM2 overexpressing U2OS cells (**A**), MCF-7 and HEK293 cells (**B**) were grown to about 70% confluency and then starved in serum-free medium for 24 hours. DMSO, 10 nM rapamycin or 5 μM LY294002 were added to cells for 5 hours prior to serum stimulation for additional 1 hour. Total proteins were then extracted in LSAB lysis buffer and subjected to Western blot analysis with antibodies against MDM2, S6K1 and actin.

In addition, I used human prostate cancer cell line PC-3, which is $p53^{-/-}$ and expresses defective form of tumor suppressor PTEN. The PI3K pathway in this cell line is constitutively active, as mutated PTEN is unable to dephosphorylate/inactivate $PI(3,4,5)P_3$, the key downstream messenger of PI3K activity. By using this cellular model, I were interested to find out whether or not treatment of PC3 cells with rapamycin could overcome positive regulatory signalling to MDM2 from PI3K/PKB pathway. Indeed, serum-induced accumulation of MDM2 protein in PC3 cells was completely abolished by treatment with 10 nM or 100 nM rapamycin (Figure 5.3). Immunoblotting with pS240/244-S6 and pT389-S6K1 antibodies confirmed the inhibitory effect of rapamycin on the mTOR/S6K pathway. Furthermore, probing with pS166-MDM2 antibody showed that phosphorylation of MDM2 at Ser166 was hardly detectable in rapamycin-treated cells, when compared to that in starved or serum-stimulated cells. This is an interesting observation, since rapamycin (a highly specific inhibitor of mTOR pathway) was capable of overcoming both PI3K- and serum-driven increase in MDM2 protein level, as well as the induction of Ser166 phosphorylation. Based on these findings I could propose that the increase of MDM2 protein level in response to serum stimulation correlates very well with Ser166 phosphorylation, and both events are predominantly mediated via mTOR/S6K pathway. The reproducibility of presented data was confirmed in at least 6 independent experiments, in which probing with anti-actin antibody was always used as a loading control.

5.2.3 The inhibitory effect of rapamycin on MDM2 protein level is dose- and time-course dependent

To reinforce the correlation between rapamycin treatment and the decline of MDM2 level, I performed a dose-course experiment with rapamycin in NIH3T3 cells. In this experiment, exponentially growing NIH3T3 cells were transfected with pcDNA3.1-MDM2-WT construct using LipofectAMINE-2000. After

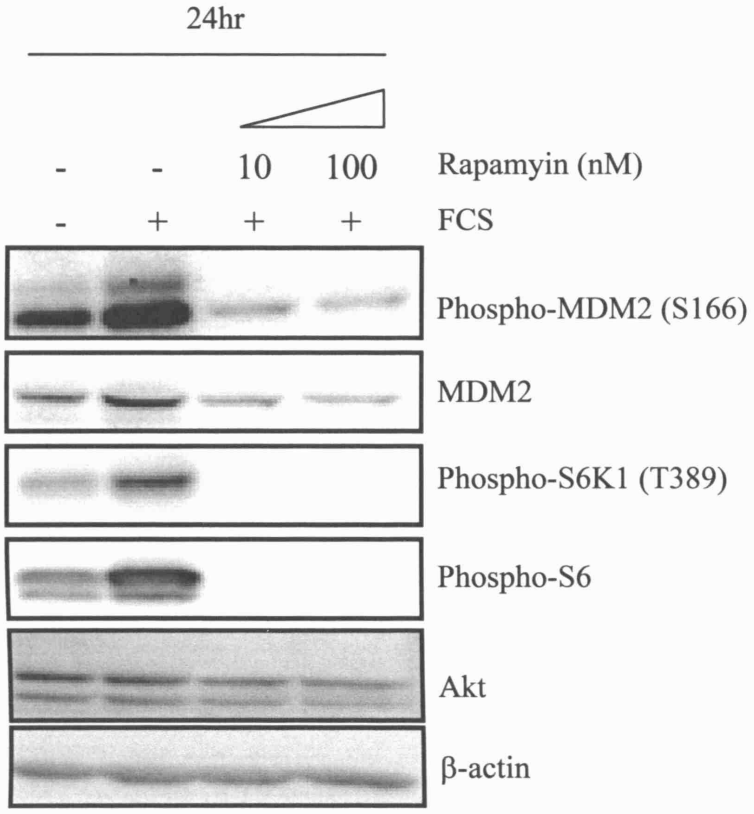


Fig. 5.3 Rapamycin blocks serum-induced increase of MDM2 protein and the phosphorylation of Ser166 in PC-3 cells.

Exponentially growing PC-3 cells were serum-starved for 16 hours and then stimulated with 10% FCS in the presence or absence of rapamycin (10 nM or 100 nM). Cells were harvested in LSAB buffer and total cellular proteins resolved by SDS-PAGE. After the transfer to the PVDF membrane, immunoblot analysis with a panel of antibodies was carried out.

serum starvation for 24 hours, cells were incubated with DMSO or various concentrations of rapamycin (1, 10, 50, and 200 nM) for 5 hours. Then, cells were stimulated with 10% donor calf serum (DCS; Life Technologies, Inc.) for 1 hour and protein lysates analyzed by immunoblotting with a panel of antibodies (anti-MDM2, anti-S6K1, anti-phospho T389 S6K1, anti-β-actin). The results presented in Figure 5.4A clearly demonstrate that the increase in rapamycin concentration draws a parallel with the relative decrease in MDM2 protein level. The densitometric analysis of the immunoreactive signals from the anti-MDM2 Western blot showed that, treatment of cells with 1 nM rapamycin resulted in approximately 10% reduction of MDM2, while the addition of 200 nM almost brought a halt to serum-induced accumulation of MDM2 (Figure 5.4B). Probing the membranes with anti-S6K1 and pT412 antibodies showed that the rapamycin-driven reduction of MDM2 protein level correlates very well with the inhibition of S6K activity.

A time-course experiment was also carried out in NIH3T3 cells, which were transiently transfected with pcDNA3.1-MDM2-WT plasmid. Twenty-four hours after transfection, cells were serum-starved and incubated with DMSO or 10 nM rapamycin for various period of time (15, 30, 120, or 300 minutes) followed by serum stimulation. Immunoblot analysis with S6K1 and pT389 S6K1 antibody demonstrates that even a short incubation (15 minutes) of cells with 10 nM rapamycin completely eliminates serum-induced activation of S6K (Figure 5.5A). Notably, the presence of multiple immunoreactive bands in anti-S6K1 blot represents multiple phosphorylation forms of S6K1. The stimulation of cells with growth factors or serum causes the accumulation of low mobility form of S6K1, which corresponds to the fully phosphorylated/activated kinase. Treatment of cells with rapamycin results in dephosphorylation of S6K1 and subsequently the appearance of a fast-migrating band in immunoblots. Immunoblotting with anti-MDM2 antibody showed that treatment of cells with rapamycin for 30 minutes and longer, eliminates the serum-induced increase of MDM2 protein and brings it within a comparable level of starved cells (Figure

5.5A and B). Notably, 15 minutes incubation of cells with rapamycin fully blocks S6K1 activity, but the level of MDM2 protein is only reduced by approximately 25%. The time which is required for proteasomal-driven degradation of MDM2 could provide a possible explanation for the difference observed (approximately 15 minutes) between full inactivation of S6K1 and the elimination of a serum-induced increase of MDM2 in response to rapamycin.

In parallel, I performed time- and dose-course analyses of LY294002 and the effect of which on MDM2 protein level. In this study, I used the same experimental procedure as that described above for rapamycin experiments. As shown in Figure 5.6A, treatment of NIH3T3 cells with various concentrations of LY294002 (1, 5, 20 and 100 μM) for 5 hours before serum stimulation had a different effect on the level of MDM2. While 5 μM and 20 μM LY294002 inhibited serum-induced accumulation of MDM2, treatment of cells with 1 μM increased the MDM2 level slightly, and 100 μM LY294002 did not change MDM2 levels at all. In previous studies, it was shown that IC50 for LY294002 inhibition of PI3K (Class 1A) is 5 μM (Krystal *et al.*, 2002). Furthermore, at this concentration LY294002 does not inhibit other protein kinases. For this reason, I tested the effect of 5 μM LY294002 on MDM2 in a time-course study. Here, NIH3T3 cells were transiently transfected with pcDNA3.1-MDM2-WT, serum-starved and incubated with DMSO or 5 μM LY294002 for 15, 30, 120 or 300 minutes. After serum stimulation, immunoblot analysis of total cell lysates with MDM2 and β-actin antibodies was carried out. As shown in Figure 5.6B, the increase in incubation time with LY294002 correlates with the gradual decrease of serum-induced MDM2 protein level. For example, treatment of cells for 5 hours brings the level of serum-driven accumulation of MDM2 to that which is observed in serum-starved cells.

In summary, I found, in various cell lines and under different experimental conditions that rapamycin blocks efficiently on the whole, but also has the ability to work particularly well to halt the accumulation of MDM2 protein in response to serum stimulation.

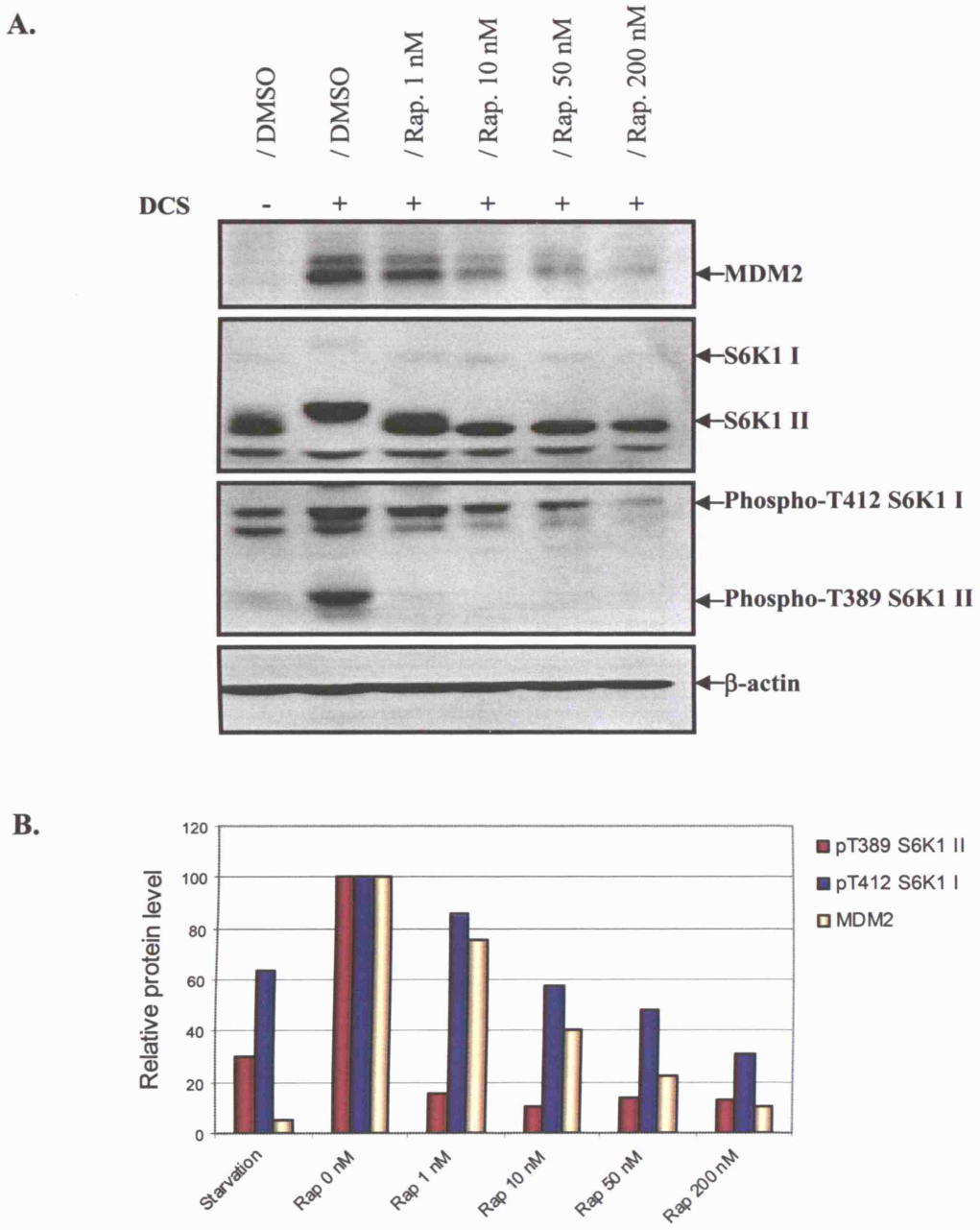


Fig. 5.4 Rapamycin reduces MDM2 protein level in a dose-dependent manner.

A. NIH3T3 cells were transfected with MDM2 expressing plasmid and starved for 24 hours. Following incubation with different concentrations of rapamycin or DMSO for 5 hours, cells were stimulated with 10% DCS for additional 60 minutes. Cells were then lysed in LSAB lysis buffer and total proteins (50 μg) were subjected to Western blot analysis with antibodies specific for MDM2, S6K1 and pT412-S6K1. **B.** A graph of the MDM2 protein signal and the level of pT412-S6K1 quantified by densitometry and normalised to the amount of protein present in each samples (based on the β-actin signal).

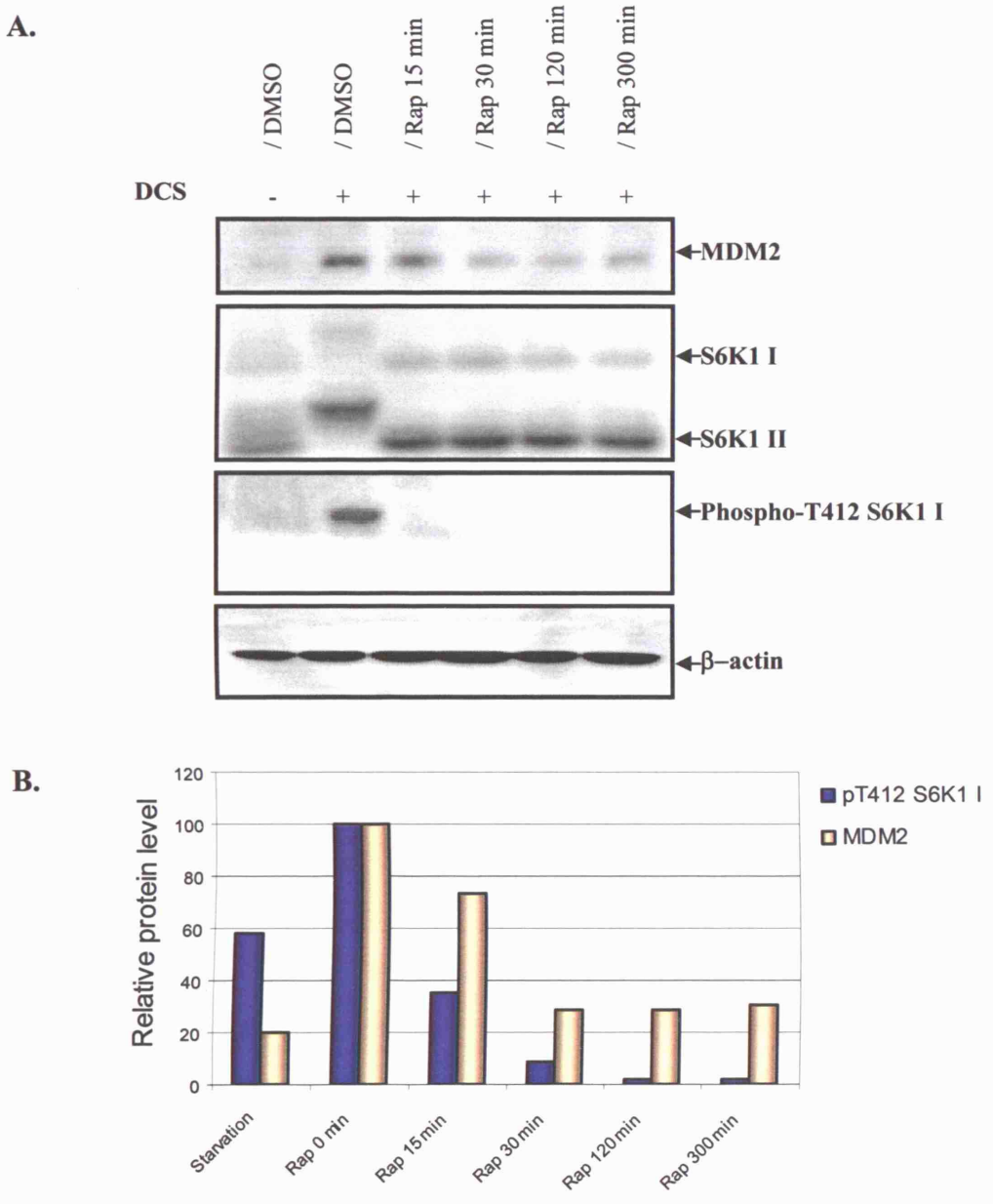


Fig. 5.5 Rapamycin reduces MDM2 protein level in a time-dependent manner.

A. NIH3T3 cells were transfected with MDM2 expressing plasmid and starved for 24 hours. Serum-starved cells were incubated in the presence of 10 nM rapamycin or DMSO for various period of time (15, 30, 120, 300 minutes) and then stimulated with 10% DCS for additional 60 minutes. After lysing in LSAB buffer, total proteins (50 μg) were separated by SDS-PAGE and subjected to Western blot analysis with antibodies specific for MDM2, S6K1 and pT412-S6K1. **B.** A graph of the MDM2 protein signal and the level of pT412-S6K1 quantified by densitometry and normalised to the amount of protein present in each samples (based on the β-actin signal).

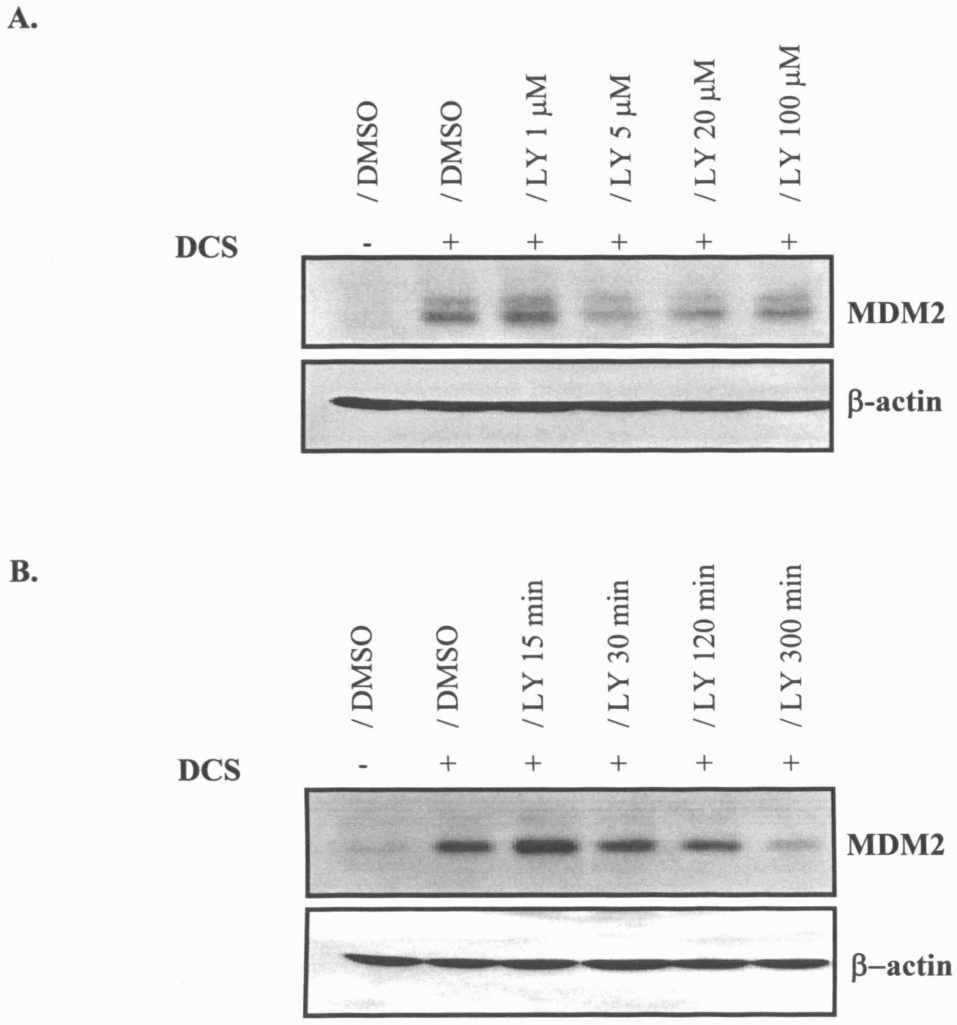


Fig. 5.6 Dose- and time-course effect of LY294002 on MDM2 level in serum stimulated NIH3T3 cells.

NIH3T3 cells were transfected with MDM2 and starved for 24 hours after transfection. Cells were incubated with various concentrations of LY294002 (1, 5, 20 and 100 μM) for 5 hours (**A**) or with a constant concentration of LY294002 (5 μM) for different periods of time (15, 30, 120 and 300min) (**B**). Then, cells were stimulated with 10% DCS for 1 hour. Total cell lysates were extracted with LSAB lysis buffer, separated on SDS-PAGE and transferred to nitrocellulose membrane. The expression of MDM2 and β-actin was analysed by immunoblotting with specific antibodies.

5.2.4 Rapamycin reduces MDM2 protein half-life

To study the mechanism by which rapamycin affects the level of MDM2 in cells, I used translational inhibitor cycloheximide (CHX). This inhibitor has been used extensively to measure the half-life of many cellular proteins, including MDM2. Feng and colleagues reported that the PI3K/PKB pathway stabilizes MDM2 protein by blocking its degradation via the proteasomal pathway (Feng et al., 2004). In MCF-7 cells, which express a high level of endogenous MDM2, I showed with the use of cycloheximide that MDM2 half-life is approximately 3 hours (data not shown). Furthermore, I used this cell line to measure MDM2 half-life in the presence or absence of rapamycin or LY294002. In this experiment, exponentially growing MCF-7 cells (at appr 70% confluency) were incubated for 5 hours with either 50 nM rapamycin, 10 M of LY294002 or DMSO. Cells were then treated with or without cycloheximide (CHX, 80 μg/ml) for the indicated times. Total proteins were extracted with LSAB lysis buffer and the clear lysates collected following centrifugation. Forty micrograms of cell lysates were separated by SDS-PAGE and subjected to Western blot analysis with anti-MDM2 antibody. As shown in Figure 5.7A, both LY294002 and rapamycin significantly reduced the level of MDM2 protein in the presence of cycloheximide. Densitometric analysis indicates that two hours of cycloheximide treatment decreases MDM2 protein by 25% in control cells, whereas the same treatment decreases MDM2 protein by 45% in the presence of rapamycin or LY294002, indicating that the degradation rate of MDM2 protein is greater in rapamycin- and LY294002-treated cells. Furthermore, the half-life of MDM2 protein in the presence of LY294002 and rapamycin was reduced to approximately 60 minutes. My data not only confirm previous reports that LY294002 accelerates protein degradation of MDM2 (Ashcroft et al., 2002;Feng et al., 2004), but also reveals the role of mTOR/S6K pathway in the regulation of MDM2 stability. This experiment was repeated three times, with reproducible results. In a control experiment, I showed that MDM2 protein was maintained at a stable level in cells which were not incubated with cycloheximide, rapamycin or LY294002 (Figure 5.7A, upper panel).

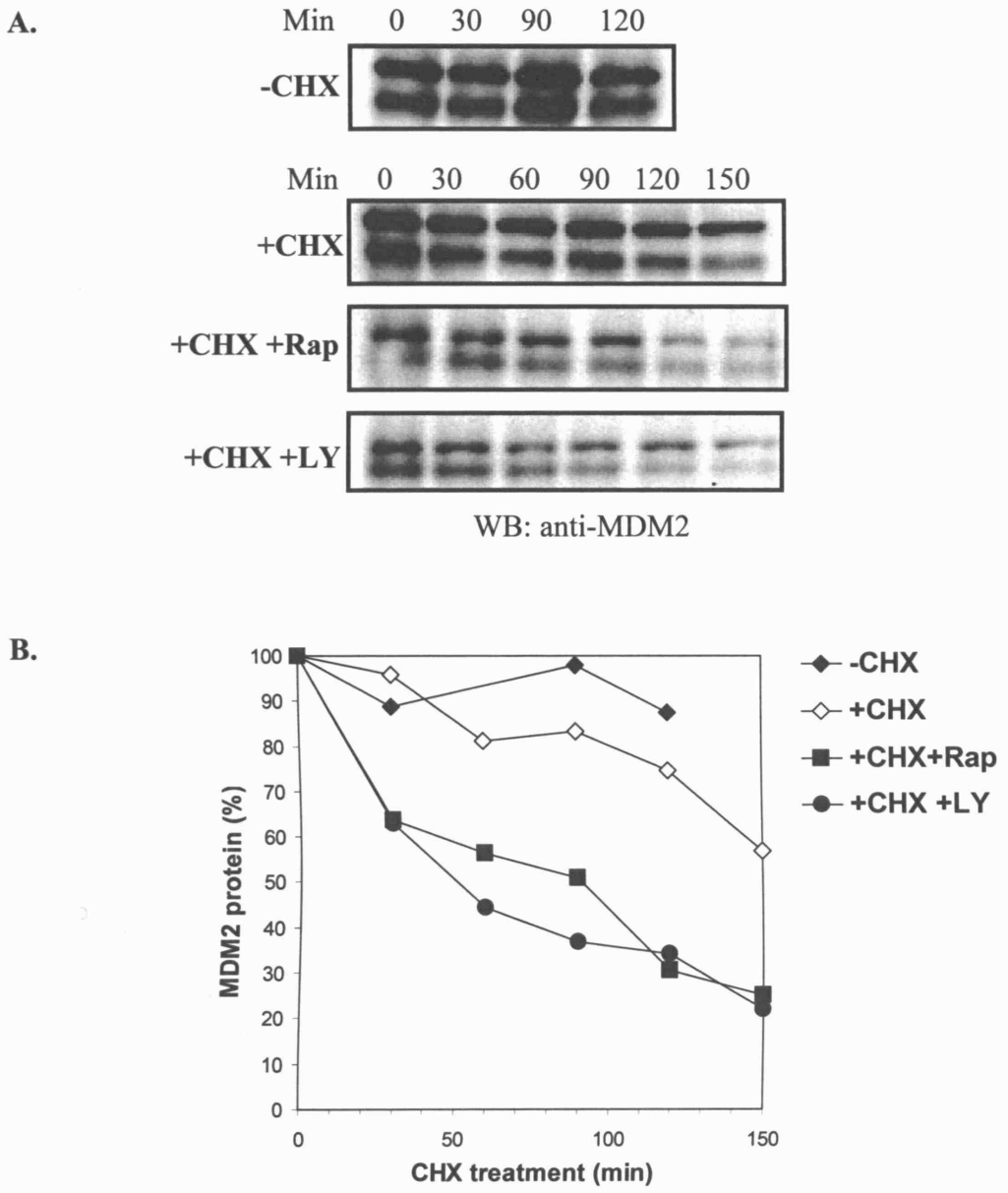


Fig. 5.7 Rapamycin reduces the half-life of MDM2 protein.

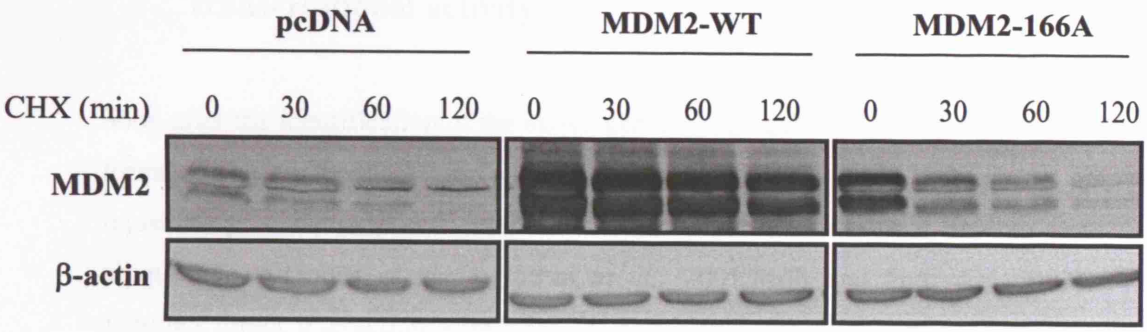
A. MCF-7 cells were seeded in 60 mm plates with DMEM medium supplemented with 10% FBS. After reaching 70% confluency, cells were incubated with either signalling inhibitors (50 nM rapamycin or 10 μM LY294002) or DMSO for 5 hours. Cells were then treated with or without cycloheximide (CHX, 80 μg/ml) for the time indicated and lysed in LSAB lysis buffer. The cell extracts were subjected to Western blot analysis with anti-MDM2 antibody to access protein expression. **B.** The level of MDM2 protein was quantified by densitometry and shown as a relative value to the MDM2 amount without cycloheximide treatment under each condition.

5.2.5 Substitution of Ser166 to Ala reduces MDM2 half-life

The above results suggest that S6K activity might be involved in stabilizing MDM2. This finding prompted us to investigate whether the phosphorylation state of serine 166, the S6K-mediated phosphorylation site in MDM2, could regulate the stability of MDM2 protein. In this study, I used the wild type MDM2 and the non-phosphorylatable mutant at position 166 (Ser to Ala substitution). The cell line PC3 was used in these experiments, as it can be transfected with much higher efficiency than the MCF-7 cell line. The PC-3 cells were plated in 6-well plates and grown in RPMI medium for 24 hours. Cells were transfected with 3 μg of one of three plasmids: wild-type MDM2 (MDM2-WT), S166A-MDM2 mutant (MDM2-166A), or empty vector (pcDNA3.1). Forty-eight hours after transfection, cells were treated with or without cycloheximide (100 μg/ml) for 30, 60, or 120 minutes. Total protein extracts were separated by SDS-PAGE and subjected to Western blot analysis with anti-MDM2 antibody. When the level of MDM2 protein was analyzed densitometrically, I found that both endogenous and transiently overexpressed MDM2 have very similar degradation rates and a half-life of approximately 3 hours (Figure 5.8A and B). Notably, the degradation rate of MDM2-166A mutant was significantly faster. For example, two hours of cycloheximide treatment reduced wild-type MDM2 protein by 30%, whereas the same treatment caused 90% reduction of MDM2-166A, indicating that the phosphorylation state of Ser166 plays a crucial role in regulating MDM2 stability. The half-life of the MDM2-166A mutant was approximately 30 minutes. This experiment was repeated three times, each producing similar results.

In summary, these data underscore the importance of Ser166 phosphorylation in the regulation of MDM2 stability and the crucial role of mTOR/S6K pathway in mediating MDM2 phosphorylation at Ser166 in response to mitogenic stimuli and nutrients.

A.



B.

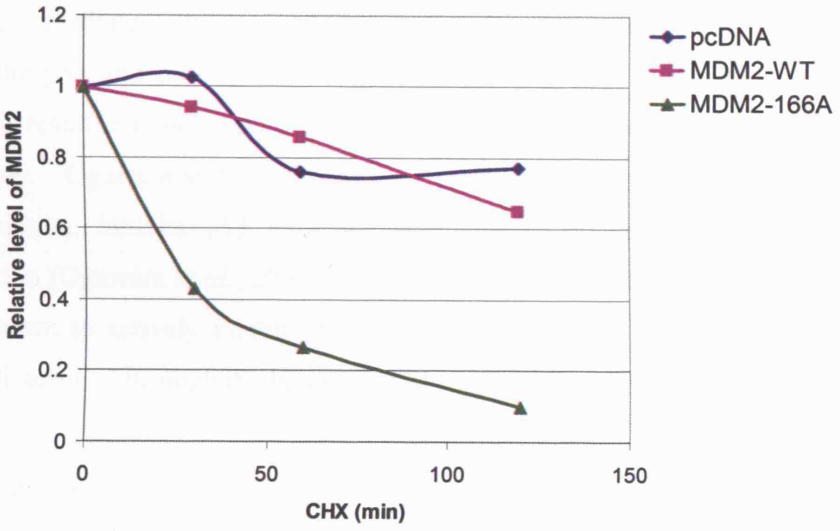


Fig. 5.8 MDM2-166A mutant is less stable than wild-type MDM2.

A. PC-3 cells were seeded in 6-well plates and transfected with 3 μg of either empty vector (control), MDM2-WT, or MDM2-166A plasmid using ExGen500 *in vitro* transfection reagent according to the instruction provided. Forty-eight hours after transfection, cells were treated with or without cycloheximide (CHX, 100 μg/ml) for the indicated time and lysed. Protein concentration of lysates were analysed with Bradford protein assay and 50 μg of proteins were subjected to Western blot analysis with antibody against MDM2. **B.** The amount of MDM2 was quantified by densitometry and standardized with the value of actin. The relative level of MDM2 was presented in comparison with the MDM2 value without cycloheximide treatment (0 minute) under each condition.

5.2.6 mTOR/S6K signalling to MDM2 does not regulate p53 transcriptional activity

Soon after the identification of the *mdm2* gene, it was realized that MDM2 protein forms a negative feedback loop which tightly regulates p53 tumor suppressor. It represses p53 transcriptional activity via two mechanisms: direct binding and ubiquitination (Jones *et al.*, 1995;Lai *et al.*, 2001;Moll and Petrenko, 2003). Through direct interaction with p53, MDM2 blocks the transcriptional activity domain of p53. (Moll and Petrenko, 2003). Through its E3 ubiquitin ligase, together with the p300 transcriptional co-activator protein (in its capacity as an E4 ligase), MDM2 mediates the ubiquitination and proteasome-dependent degradation of the p53 and other growth regulatory proteins (Lai *et al.*, 2001). Both mechanisms result in a reduction of both p53 transcriptional activity, and the apoptosis pathway. Ogawara and colleagues have reported that active PKB/Akt or serum stimulation, inhibits p53 transcriptional activity by enhancing p53 protein degradation (Ogawara *et al.*, 2002). Phosphorylated MDM2 at the serine 186 has been shown to actively inhibit p53 functions, resulting in a subsequent promotion of cell survival through the blocking of the apoptotic pathway.

To find out whether or not S6K-mediated phosphorylation of MDM2 at Ser166 affects p53 transcriptional activity I employed a p53 reporter assay in PC3 cells, which are deficient for p53. Initially, I tested the effect of rapamycin on p53 activity in the presence of wild-type MDM2 (MDM2-WT) or S166A-MDM2 mutant (MDM2-166A). I would like to thank Dr. M. Tarunina for providing p53 expression and CAT-reporter plasmids. In this experiment, PC-3 cells were seeded in 60 mm culture dishes and maintained in RPMI medium for 24 hours prior to transfection. Cells were then co-transfected with 25 ng of p53 plasmid and 1 μg of p53 CAT-reporter, together with 3 μg of one of the following mammalian expression plasmids: MDM2-WT, MDM2-166A, or the empty vector (pcDNA3.1). Twenty-four hours after transfection, cells were treated with either 50 nM rapamycin or DMSO for an additional 24 hours. The lysates were

prepared and subjected to the CAT-activity measurement as described in Material and Methods section, 2.2.6.3.1. All the measurements were standardized by protein concentration in each sample.

In line with previous studies (Alarcon-Vargas and Ronai, 2002;Moll and Petrenko, 2003), wild-type MDM2 strongly inhibited p53 transcriptional activity (Figure 5.9). I found that CAT activity in the MDM2-WT transfected cells was decreased 10-fold when compared with control pcDNA3.1 transfected cells. However, my data shows that the substitution of serine 166 to alanine in MDM2 does not affect its inhibitory activity towards p53-directed transcription. Phosphorylation at this site may therefore not be important for the negative regulation of p53 transcriptional activity.

Notably, the presence of rapamycin did not significantly affect p53 transcriptional activity in cells transfected with vector alone, indicating that the mTOR/S6K pathway does not signal to p53-mediated transcription. Furthermore, rapamycin did not reverse the inhibitory effect of MDM2-WT or MDM2-166A on p53 transcriptional activity. These results clearly show that S6K-driven phosphorylation of MDM2 at Ser166 does not contribute to the regulation of p53 transcription

In order to validate the results presented above, I employed a different experimental setup. I performed a p53 reporter assay in the presence of activated forms of PKB (PKB-CAAX), S6K1-CA (T389E) and S6K2-CA (T388E). PC3 cells were co-transfected with p53 plasmid and p53 reporter plasmid together with one of the following: MDM2-WT, PKB-CAAX, S6K1-CA, S6K2-CA, or vector alone. The reporter assay again indicated that wild-type MDM2 efficiently inhibits p53 transcriptional activity (Figure 5.10). The activated form of PKB (PKB-CAAX, contains a sequence which targets the kinase to the membrane, where it is stimulated by upstream signalling), also represses p53 activity by approximately 30%, which is in agreement with previous reports (Zhou *et al.*, 2001;Mayo and Donner, 2001;Ogawara *et al.*, 2002;Gottlieb *et al.*, 2002).

However, this is not the case for activated forms of S6K1 and S6K2. Both S6K1-CA and S6K2-CA did not affect p53 transcriptional activity in several independent experiments.

In summary, the use of p53-reporter assays provide evidence that mTOR/S6K signalling and the phosphorylation of MDM2 at Ser166 do not participate in the regulation of p53 transcriptional activity, but may regulate other downstream target(s).

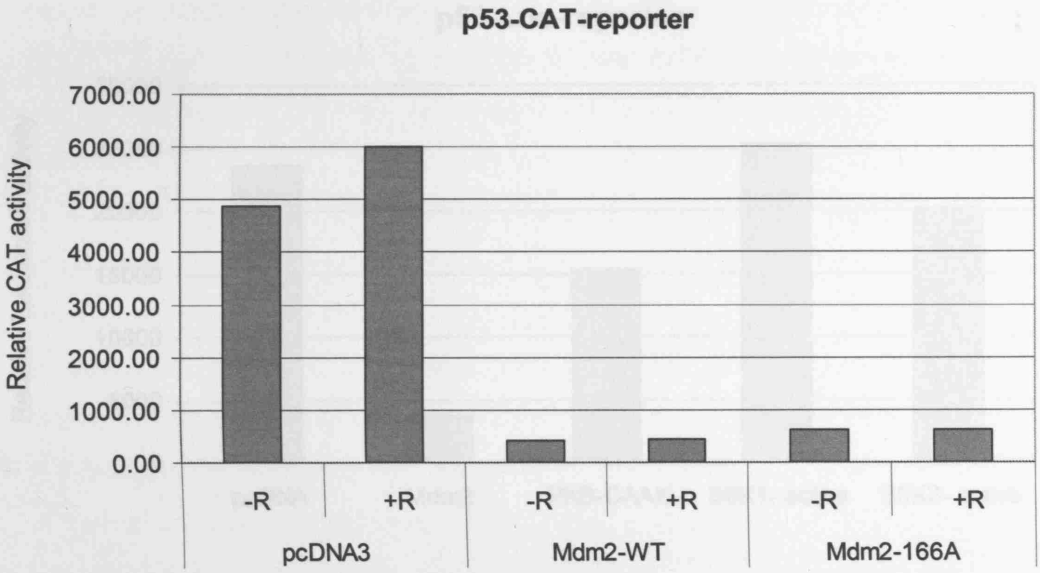


Fig. 5.9 S6K/MDM2 pathway has no effect on p53 transcriptional activity.

PC-3 cells plated in 60 mm dishes were co-transfected with p53 plasmid and p53-CAT-reporter, together with MDM2-WT, MDM2-166A, or pcDNA empty vector. Cells were treated with either rapamycin (R, 50 nM) or DMSO for 24 hours the day after transfection. Cells were then collected and the CAT activity in the cell extracts was measured as described in session 2.2.6.3.1.

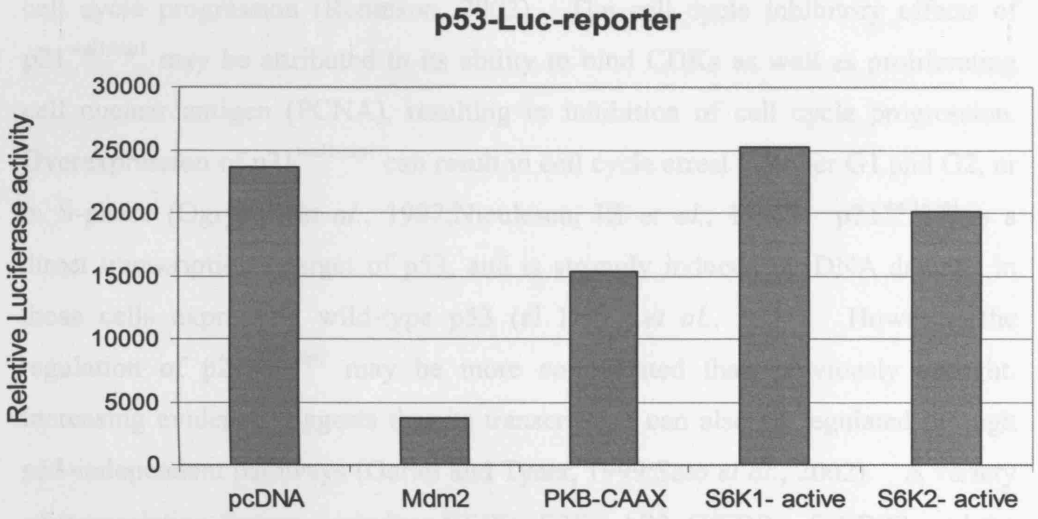


Fig. 5.10 S6K/MDM2 pathway has no effect on p53 transcriptional activity.

PC-3 cells plated in 60 mm dishes were co-transfected with p53 plasmid and p53-Luc-reporter, together with one of the following mammalian expression plasmids: MDM2-WT, PKB-CAAX, S6K1-active, S6K2-active, or pcDNA empty vector. Cells were lysed and 25 µl of the lysates were subjected to luciferase activity-calculation as described in session 2.2.6.3.2.

5.2.7 The regulation of p21 protein level involves MDM2 phosphorylation at Ser166

The CDK inhibitor $p21^{waf1/cip1}$ was initially discovered as a negative regulator of cell cycle progression (Roninson, 2002). The cell cycle inhibitory effects of $p21^{waf1/cip1}$ may be attributed to its ability to bind CDKs as well as proliferating cell nuclear antigen (PCNA), resulting in inhibition of cell cycle progression. Overexpression of $p21^{waf1/cip1}$ can result in cell cycle arrest in either G1 and G2, or in S-phase (Ogryzko *et al.*, 1997;Niculescu, III *et al.*, 1998). $p21^{waf1/cip1}$ is a direct transcriptional target of p53, and is strongly induced by DNA damage in those cells expressing wild-type p53 (el Deiry *et al.*, 1994). However, the regulation of $p21^{waf1/cip1}$ may be more complicated than previously thought. Increasing evidence suggests that its transcription can also be regulated through p53-independent pathways (Gartel and Tyner, 1999;Sato *et al.*, 2002). A variety of transcription factors, including STATs, E2Fs, AP2, C/EBPα, C/EBPβ, and the homeobox transcription factor *gax*, can regulate $p21^{waf1/cip1}$ transcription through *cis*-acting elements in the $p21^{waf1/cip1}$ promoter (Gartel and Tyner, 1999;Sato *et al.*, 2002). Although activation of $p21^{waf1/cip1}$ is important in mediating p53-dependent cell growth arrest, $p21^{waf1/cip1}$ is not essential for p53-mediated apoptosis (Deng *et al.*, 1995). Furthermore, recent studies have suggested that ubiquitination may not necessarily be a prerequisite for proteolysis of $p21^{waf1/cip1}$ by the proteasome pathway (Sheaff *et al.*, 2000).

Although $p21^{waf1/cip1}$ has been known to be degraded through the proteasome pathway, the mechanism of $p21^{waf1/cip1}$ degradation regulation in cells was still a mystery, until very recently. MDM2 was reported to promote $p21^{waf1/cip1}$ degradation via the proteasome pathway, through a direct interaction with the carboxyl-terminus of $p21^{waf1/cip1}$ (Jin *et al.*, 2003;Zhang *et al.*, 2004). This MDM2-mediated degradation is independent of both p53 and ubiquitination; as anti-MDM2 antisense oligonucleotide or short interference RNA targeting MDM2 causes significant elevation of $p21^{waf1/cip1}$ protein level in the p53-null PC3 cells

(Zhang *et al.*, 2004); and does not require the RING finger domain of MDM2, which is important for MDM2-mediated p53 degradation (Jin *et al.*, 2003). On the other hand, rapamycin has been reported to induce the expression of $p21^{waf1/cip1}$, consistent with cell cycle withdrawal (Gao *et al.*, 2003;Martin *et al.*, 2004). Furthermore, overexpression of S6K1 inhibited rapamycin-induced contractile protein and $p21^{waf1/cip1}$ expression in vascular smooth muscle cells (Martin *et al.*, 2004). All these discoveries indicate both S6K and MDM2 involvement in the regulation of $p21^{waf1/cip1}$ stability. In an attempt to find a downstream target for the S6K/MDM2 interaction, I began to investigate the possibility of $p21^{waf1/cip1}$ as an effector for S6K/MDM2 signalling. Therefore, I hypothesized that the S6K-mediated phosphorylation of MDM2 at Ser166 might regulate $p21^{waf1/cip1}$ in a p53-independent manner.

To test this hypothesis, I analyzed the inhibitory effect of rapamycin in the PC-3 human prostate cancer cell line, which is p53- and PTEN-negative. Cells were seeded in 60 mm culture dishes and maintained in RPMI medium supplemented with 10% FBS for 24 hours, followed by 16 hours of starvation in serum-free RPMI. Rapamycin (10 nM), LY294002 (5 μM), or DMSO were then added to cells in the presence or absence of 10% FCS as indicated for another 24 hours. The total cellular protein extracts were prepared and subjected to Western blot analysis using specific antibodies.

As shown in Figure 5.11, both inhibitors, especially rapamycin, inhibit Ser166 phosphorylation and S6K1/2 activation. In agreement with previous studies, LY294002 inhibits phosphorylation/activation of PKB/Akt at Ser473, while serum stimulation and rapamycin induce phosphorylation/activation. Probing with anti-p21 antibody indicated that treatment of cells with rapamycin elevated $p21^{waf1/cip1}$ protein level, a result which is in agreement with published data (Gao *et al.*, 2003). The increase in $p21^{waf1/cip1}$ protein level following rapamycin treatment was reproduced in several independent experiments. In addition, the amount of protein loaded from each sample was confirmed as equal using immunoblot analysis with anti-β-actin antibody.

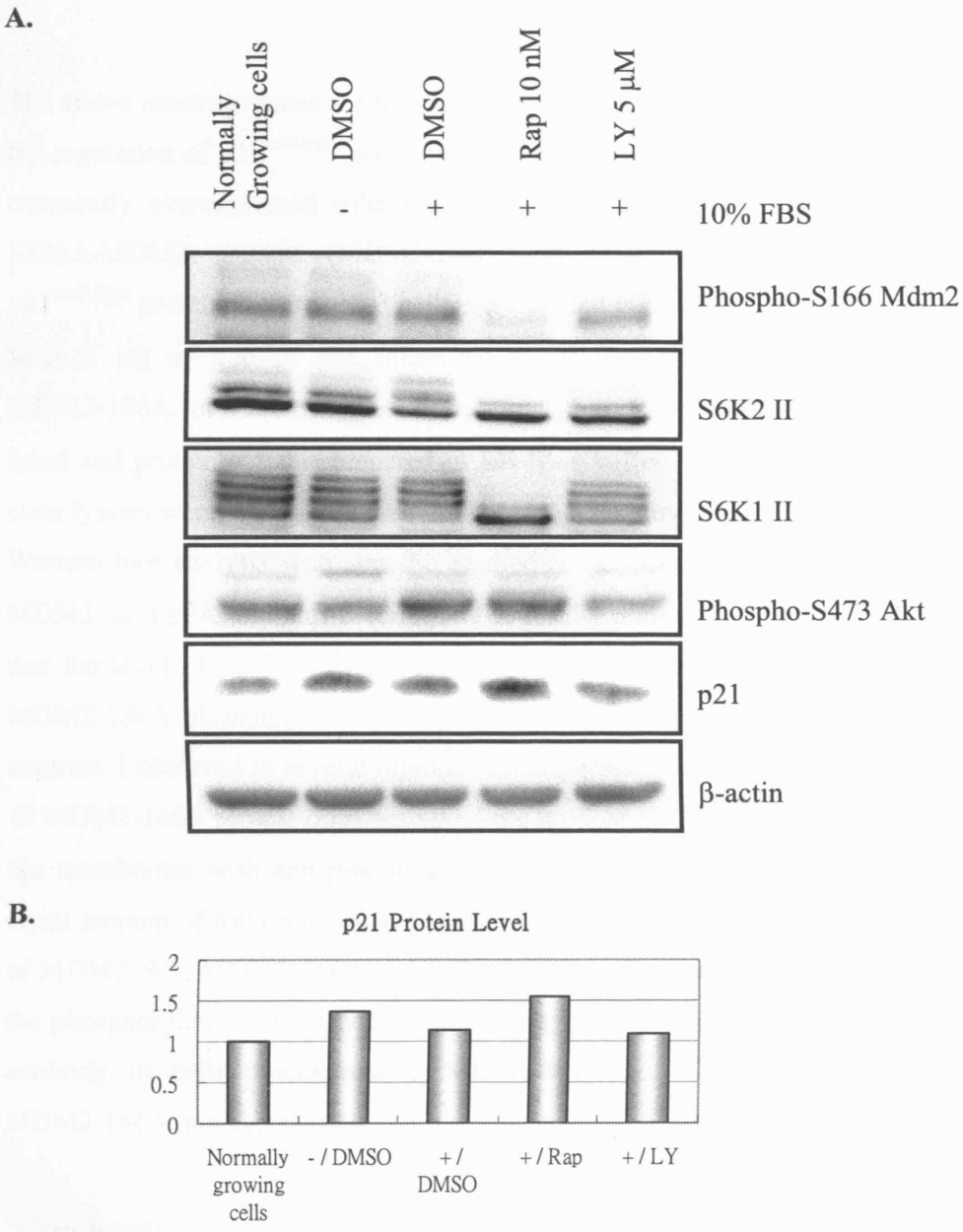


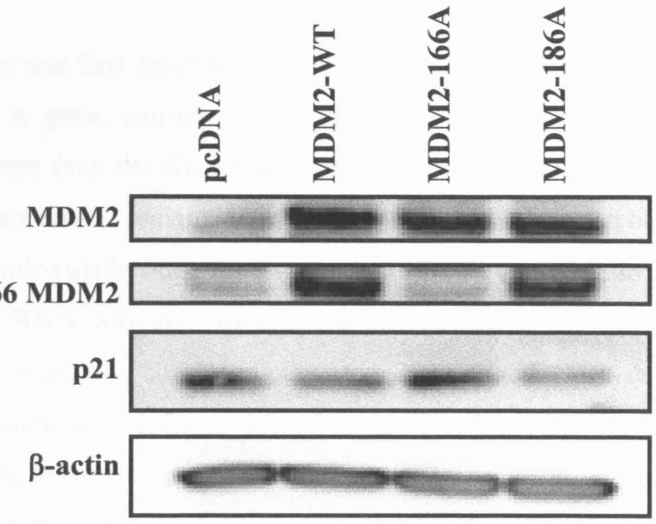
Fig. 5.11 The effect of rapamycin and LY294002 on the level of p21 and the phosphorylation status of Ser166 in PC-3 cells.

A. PC-3 cells (p53 and PTEN negative) were cultured in 60 mm dishes with RPMI medium supplemented with 10% FBS for 24 hours prior to 16 hours of serum-starvation. Cells were switched to the media in the presence or absence of 10% FBS, rapamycin (10 nM), and LY294002 (5 μM) as indicated for additional 24 hours. The total cellular proteins were extracted and subjected to Western blot analysis using specific antibodies. **B.** The protein levels of p21 were quantified by densitometry and standardized with the blot of β-actin. Data was presented as a relative value to that of normally growing cells. This experiment was repeated at least three times and each of them has presented similar result.

The above results promted me to investigate the role of Ser166 phosphorylation in the regulation of $p21^{waf1/cip1}$ protein level. In this study, I analyzed the effect of transiently overexpressed wild-type MDM2 (MDM2-WT), S166A-MDM2 and S186A-MDM2 mutants (MDM2-166A and MDM2-186A, respectively) on $p21^{waf1/cip1}$ protein. Here, PC-3 cells were seeded in 6-well plates and transfected with 3 μg of one of the following plasmids: MDM2-WT, MDM2-166A, MDM2-186A, or vector alone. Forty-eight hours after transfection, cells were lysed and protein extracts prepared in LB lysis buffer. Fifty μg of each of the clear lysates were loaded in a gradient gel (4%-12%, Invitrogen), and subjected to Western blot analysis with specific antibodies (anti-MDM2, anti-phospho S166 MDM2, anti-p21, and anti-β-actin). The results presented in Figure 5.12 show that the level of $p21^{waf1/cip1}$ is reduced in cells transfected with MDM2-WT or MDM2-186A plasmids, when compared to the level in a control sample. In contrast, I observed in several reproducible experiments, that transient expression of MDM2-166A protein does not affect the level of $p21^{waf1/cip1}$ protein. Probing the membranes with anti-β-actin and anti-MDM2 antibodies confirmed that an equal amount of total protein was loaded on the gel and that transient expression of MDM2-WT, MDM2-166A and MDM2-186A was very similar. As expected, the phosphorylation of MDM2 at Ser166 was clearly detectable with anti-pS166 antibody in cells overexpressing MDM2-WT and MDM-186A, but not in MDM2-166A transfected cells.

Taken together, my data strongly indicate that S6K-mediated phosphorylation of MDM2 at Ser166 contributes to the regulation of $p21^{waf1/cip1}$ protein levels and that this process is independent of the p53 signalling pathway.

A.



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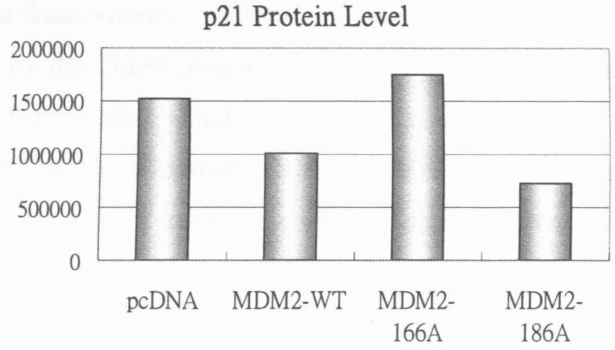

Fig. 5.12 MDM2-166A mutant does not affect the level of p21, when compared to the MDM2-WT.

A. The $p53^{-/-}$ PC-3 cells were transfected with MDM2-WT, MDM2-166A, MDM2-186A, or pcDNA 3.1 empty vector for 48 hours. Cells were then lysed in LSAB lysis buffer and the total proteins (80 μg) were subjected to Western blot analysis. The blot was probed with specific antibodies to assess the protein level of MDM2, phospho-MDM2 at Ser166, p21, and β-actin. **B.** The protein levels of p21 were quantified by densitometry and standardized with the blot of β-actin. Data was presented as a relative value to the control. This experiment was repeated four times and each of them has given similar result, though the presented figure is one of the best result.

5.2.8 RNA interfering knockdown studies implicate S6K2, but not S6K1 in MDM2 phosphorylation at S166

RNA interference was first described in plants in 1990 when attempts to introduce extra copies of a gene, caused a decrease in its expression rather than the anticipated increase (van der Krol *et al.*, 1990;Napoli *et al.*, 1990). For several years, this phenomenon remained an oddity until the larger community of developmental biologists became involved. In 1998, Fire and Mello showed that double stranded RNA was able to direct the degradation of messenger RNA (mRNA) with a sequence complementary to either strand (Fire *et al.*, 1998). This key observation solved the problem brought to light by previous groups in 1990, and also led to the introduction of the term "RNA interference." Over the last decade, the mechanism underlying RNA interference has been established from work carried out on a range of organisms, especially the worm and the fruit fly. The process of RNA interference was considered to be a mechanism for protecting organisms from viruses. Briefly, double-stranded RNA molecules in the cell are cleaved by the Dicer enzyme complex to form small interfering (si) double-stranded RNA molecules, which are some 20 bases pairs long. These are recognized by another enzyme complex, the RNA-induced silencing complex (RISC), which uses one strand to target complementary mRNA molecules for degradation.

Owing to its high specificity and speed of action, the power that RNA interference promised has been recognized in the fields of both biochemical and biomedical research. By directing a specifically designed double-stranded RNA into cells, researchers are now able to seek out a specific gene which has been targeted for "knock-down." However, initial attempts to induce the RNA interference response in mammalian cells were unsuccessful. Introduction of double-stranded RNA in mammalian cells induces an antiviral response involving the production of interferon, which results in the total inhibition of gene expression and rapid cell death. The breakthrough came when short,

double-stranded RNA molecules of less than 30 nucleotide pairs long were shown to be capable of directing a sequence specific degradation of homologous mRNA in mammalian cells without inducing the interferon response (Elbashir *et al.*, 2001). Small interfering RNAs (siRNAs) of about 20 nucleotide pairs in length can specifically repress the expression of a gene with a given sequence in mammalian cells, and have been developed and are now in use as a powerful tool in biochemical and biomedical research.

With the increase in data being published in the literature with regards to the use of siRNAs against S6Ks, I have tried to establish the system in the hope of identifying any functional differences between S6K1 and 2 in the regulation of MDM2. Double-stranded small interfering RNAs (siRNAs) against S6K1 (5'-GGACAUGGCAGGAGUGUUUTT-3') and S6K2 (5'-GGACCAAGAAGUCCAAGAGTT-3') were manufactured by MWG Biotech AG, based on a previous report (Harrington *et al.*, 2004). Preliminary experiments were performed in order to demonstrate the efficiency of the synthetic siRNAs in cultured mammalian cells. To do this, HEK 293 cells were seeded in 60 mm tissue culture dishes with the density of 25×10^4 cells per dish. Four hundred pM of each siRNA, including a non-specific, scrambled sequence control siRNA, were transfected into cells using LipofectAMINE^{TM} 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. Cells were harvested 4 days post-transfection and total cellular proteins were extracted with EB lysis buffer. The clear lysates were analyzed by Western blotting with antibodies against either S6K1 or S6K2. The protein levels of S6K1 and S6K2 were quantified by densitometry and standardized with β-actin in each lane. An 80-90% knock-down of S6K1 protein level was observed when introducing siRNA against S6K1 into HEK 293 cells, while siRNA against S6K2 inhibited cellular S6K2 level about 40-50% (Figure 5.13).

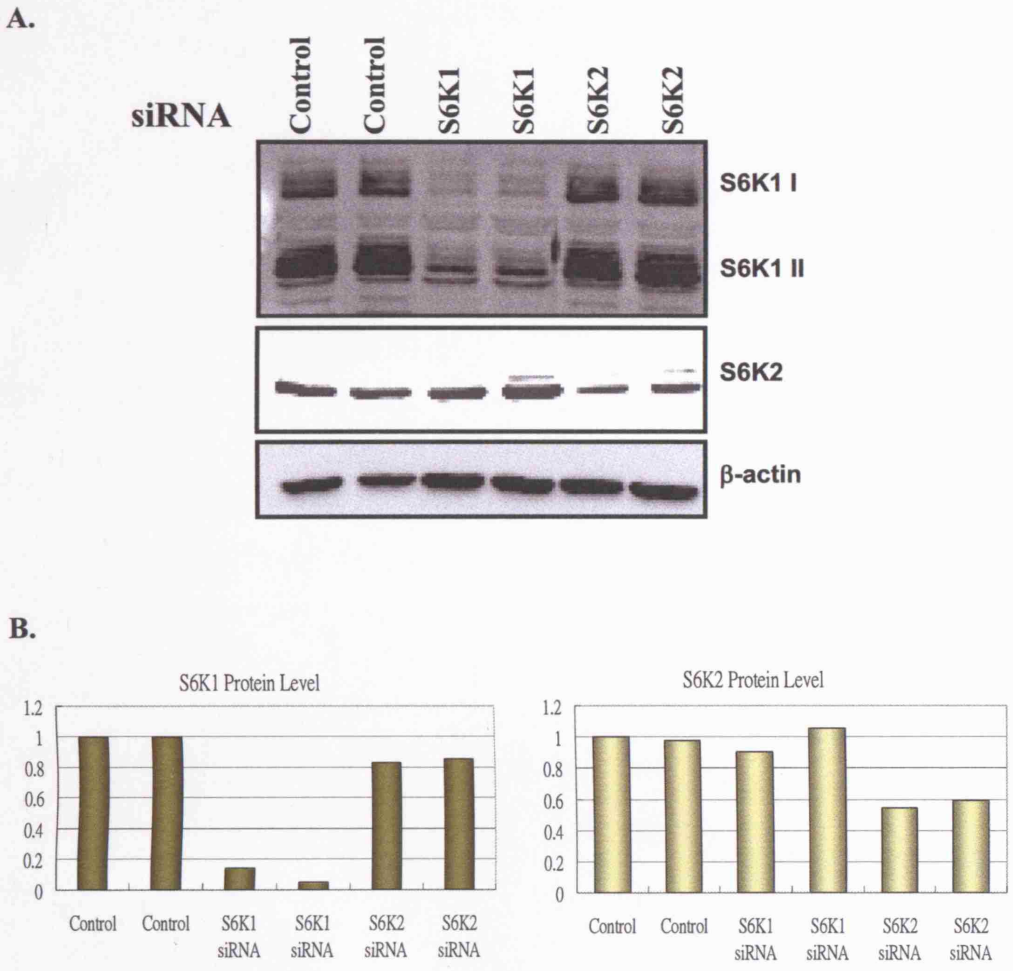

Fig. 5.13 Testing the efficiency of S6K1 and S6K2 siRNAs in HEK293 cells.

A. HEK293 cells were seeded in 60 mm culture plates with the density of 25×10^4 cells per dish. Cells were transfected with 400 pM of siRNAs against either S6K1 or S6K2, or non-specific control as indicated on the figure. Transfected cells were grown in DMEM culture media for another 4 days. Total cellular proteins were extracted with EB lysis buffer and 50 μg of each lysate was separated on SDS-PAGE and subjected to Western blotting using specific antibodies. **B.** The protein levels of S6K1 and S6K2 were quantified by densitometry and standardized with the blot of β-actin. Data was presented as a relative value to the control.

In order to verify the contribution of S6K1 and S6K2 to MDM2 phosphorylation in cells, I reduced either S6K1 or S6K2 expression by means of siRNA technology, and analyzed the effects of which on serine 166 phosphorylation. HEK 293 cell were transfected with one of the following synthetic siRNAs: S6K1, S6K2, PKB/Akt (5'-UGCCCUUCUACAACCAGGATT-3', (Katome *et al.*, 2003)), or a non-specific control, as described previously. Total cellular proteins were extracted with EB lysis buffer 4 days after transfection and subjected to Western blot analysis with specific antibodies (Figure 5.14A). The phosphorylation level of serine 166 phosphorylation was quantified by densitometry and standardized by the level of β-actin in each lane. Data was presented as a relative ratio to the control (Figure 5.14B).

As shown in Figure 5.14, PKB siRNA effectively reduced PKB expression by 50% in this experiment. However, the reduction of PKB did not decrease MDM2 phosphorylation. On the contrary, the phosphorylation of serine 166 was increased, together with the elevation of S6K activity, which can be seen from the increased S6 phosphorylation. This result confirms my previous data and also my hypothesis that PKB/Akt is not a direct regulator of MDM2 phosphorylation on serine 166 under certain circumstances, if at all.

The siRNA against S6K2 led to a significant level of inhibition of S6K2 expression and, concurrently to a potent reduction of MDM2 phosphorylation at S166 in comparison with the scrambled siRNA. The reduction in S6K2 protein expression correlates with a diminished S6K activity, as the phosphorylated S6 protein was reduced. This result associates MDM2 phosphorylation at Ser166 with S6K2-mediated signalling. In the case of S6K1 siRNA-transfected cells, I found that a significant decrease in S6K1 expression was accompanied by an elevated level of S6K2. Unlike S6K2, elimination of S6K1 resulted in a dramatic increase of phosphorylated serine 166, as well as phosphorylated S6 protein, which may be a result of the elevated S6K2, and PKB/Akt expression. This confusing result suggests there may be an internal feedback control between S6K1 and 2, since once S6K1 expression was severely repressed, S6K2

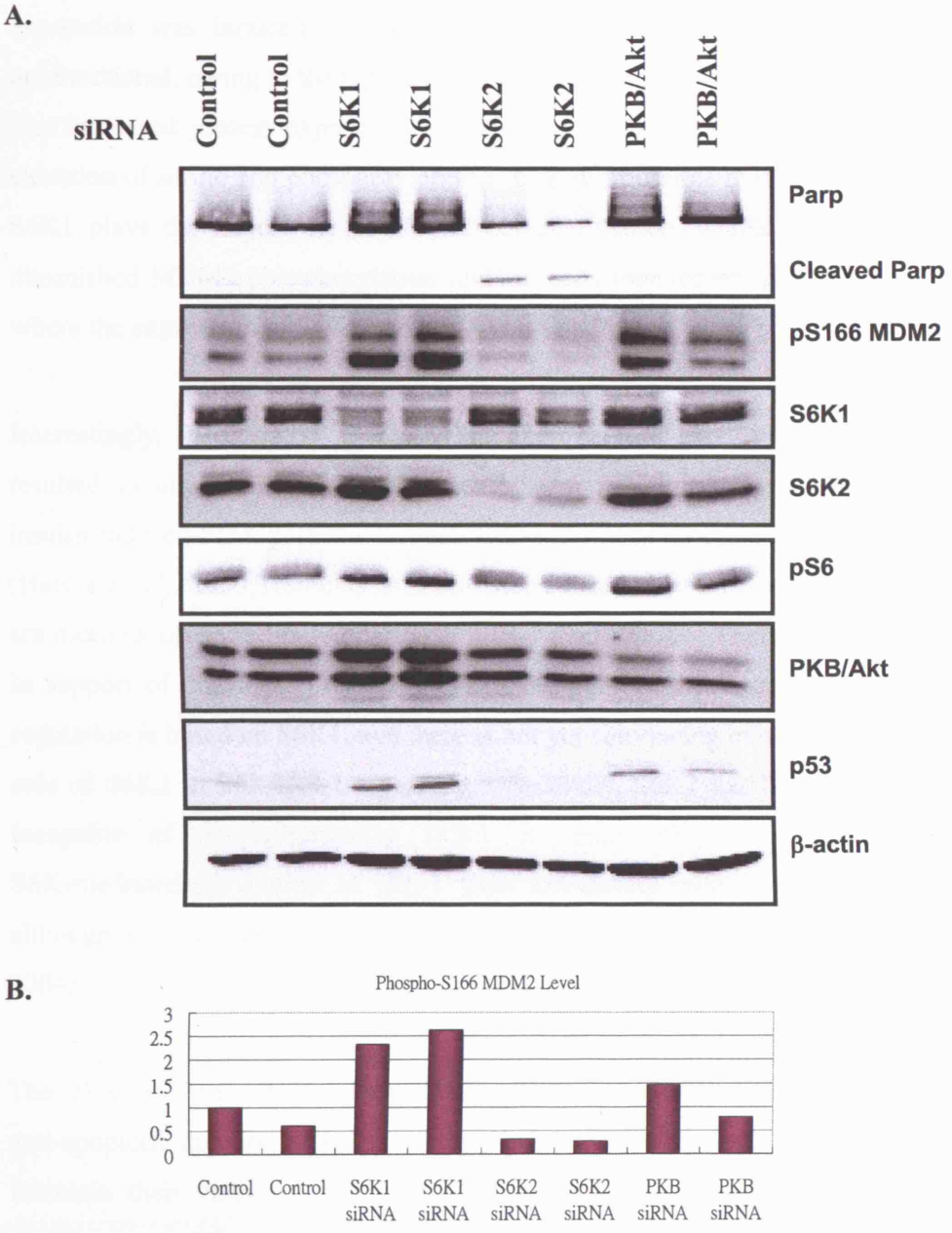

Fig. 5.14 The phosphorylation of MDM2 at Ser166 is significantly reduced by siRNAs for S6K2, but not S6K1 or PKB/Akt.

A. HEK293 cells were seeded in 60 mm culture plates with the density of 25×10^4 cells per dish. Cells were transfected with 400 pM of either S6K1, S6K2, PKB, or non-specific control siRNA as indicated in the figure, followed by incubation in DMEM culture medium for another 4 days. Total cellular proteins were extracted with EB lysis buffer and 50 μg of each lysate was separated on SDS-PAGE and subjected to Western blotting using specific antibodies. **B.** The level of phosphorylated MDM2 was quantified by densitometry and standardized with β-actin. Data was presented as a relative value to the control. The same result were observed at least in four independent experiments.

expression was induced. This feedback regulation, however, appears to be unidirectional, owing to the repression of S6K2 failing to elevate S6K1 expression. The increased protein expression and kinase activity of S6K2 may explain the elevation of serine 166 phosphorylation. It is also possible that S6K2 rather than S6K1 plays the major role in MDM2 phosphorylation, which may explain the diminished MDM2 phosphorylation level in cells transfected with S6K2 siRNA, where the expression of S6K1 or PKB/Akt was still high.

Interestingly, S6K1 siRNA increased the expression of PKB while S6K2 siRNA resulted in unaltered PKB protein level. An inhibitory role of S6K on the insulin-induced PI3K pathway through IRS-1 has been reported by several groups (Haruta *et al.*, 2000;Tremblay and Marette, 2001). My data implies that S6K1 are more involved in the regulation of IRS-1 than S6K2. There are two reasons in support of this idea. Firstly, most of the published research on S6K/IRS-1 regulation is based on S6K1, and there is not yet convincing evidence to show the role of S6K2 in S6K/IRS-1 signalling. Secondly, S6K2 has been shown to be incapable of phopsphorylating IRS-1 at serine 302, a crucial event of S6K-mediated prevention of IRS-1 from associating with its insulin receptor, although it was able to regulate IRS-1 mRNA transcription (Harrington *et al.*, 2004).

The cleaved Parp observed in S6K2 siRNA-transfected cells confirmed the anti-apoptotic function of S6K2. The importance of Parp is that it allows cells to maintain their viability; cleavage of Parp facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis (Oliver *et al.*, 1998). A previous report published by Pardo and colleagues demonstrated S6K2 to be of central importance in the regulation of cell death in mammalian cells and showed that this isoform integrates the pro-survival signalling function of FGF-2 (Pardo *et al.*, 2006). In line with this idea, my experiment showed that elimination of S6K2 caused increased cleavage of Parp, indicating an enhanced apoptosis pathway.

Next, I tested the effect of S6K2 siRNA on MDM2 stability. The siRNAs against either S6K1 or S6K2 or the non-specific control were delivered into HEK293 cells using LipofectAMINE^{TM} 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. Three days after transfection, cells were starved in serum-free DMEM medium for another 24 hours. Cells were then stimulated with or without 10% FBS one hour before harvesting. Total cellular proteins were extracted with EB lysis buffer and analyzed by Western blotting using specific antibodies (Figure 5.15A). The level of each protein was quantified by densitometry and standardized with β-actin in each lane. The chart is presented as a relative value to the control (Figure 5.15B). The MDM2 protein level was reduced by S6K2 siRNA but elevated by S6K1 siRNA (Figure 5.15). The alteration of protein level was more dramatic in serum-stimulated cells, where 50% elimination of S6K2 resulted in a 66% reduction of MDM2 protein level. Inhibition of S6K1 caused a 60% induction of MDM2 together with an elevation of S6K2 expression around 30%. Notably, the extent of the band shift I observed in S6K2 extracted from S6K1 siRNA-transfected cells suggests the presence of a more active S6K2, though the total protein level was only slightly increased. Moreover, the increased β-actin in S6K1 siRNA-transfected cells, which contained enhanced S6K2, indicates a healthier cell condition, substantiating further the probable involvement of S6K2 in survival. I have also noticed the slightly different inhibitory efficiency of the siRNAs on S6K1 and 2 protein levels through out these experiments (Figure 5.13-5.15). This is due to a variable expression of siRNAs in each experiment, on which the inhibitory efficiency of the target proteins rely.

Taken together, my experiments using siRNAs revealed different effects from the reduction of S6K1 and 2 on MDM2 regulation. The phosphorylation of MDM2 at serine 166 may be more likely to be associated with the activity of S6K2 rather than S6K1 in certain circumstances. This phosphorylation was correlated with the changes in MDM2 stability and S6K2 activity. Moreover, S6K1 siRNA exhibited a unidirectional balancing regulation between the two isoforms. Severe diminution of S6K1 resulted in the induction of S6K2 expression, but not

vice versa, which in turn caused the elevation in MDM2 phosphorylation at Ser166 and stability. In addition to the regulation of MDM2, this data also confirms previous reports showing diverted functions between the two isoforms. S6K1 may be more involved in the IRS-1 regulation, while S6K2 has an anti-apoptotic function.

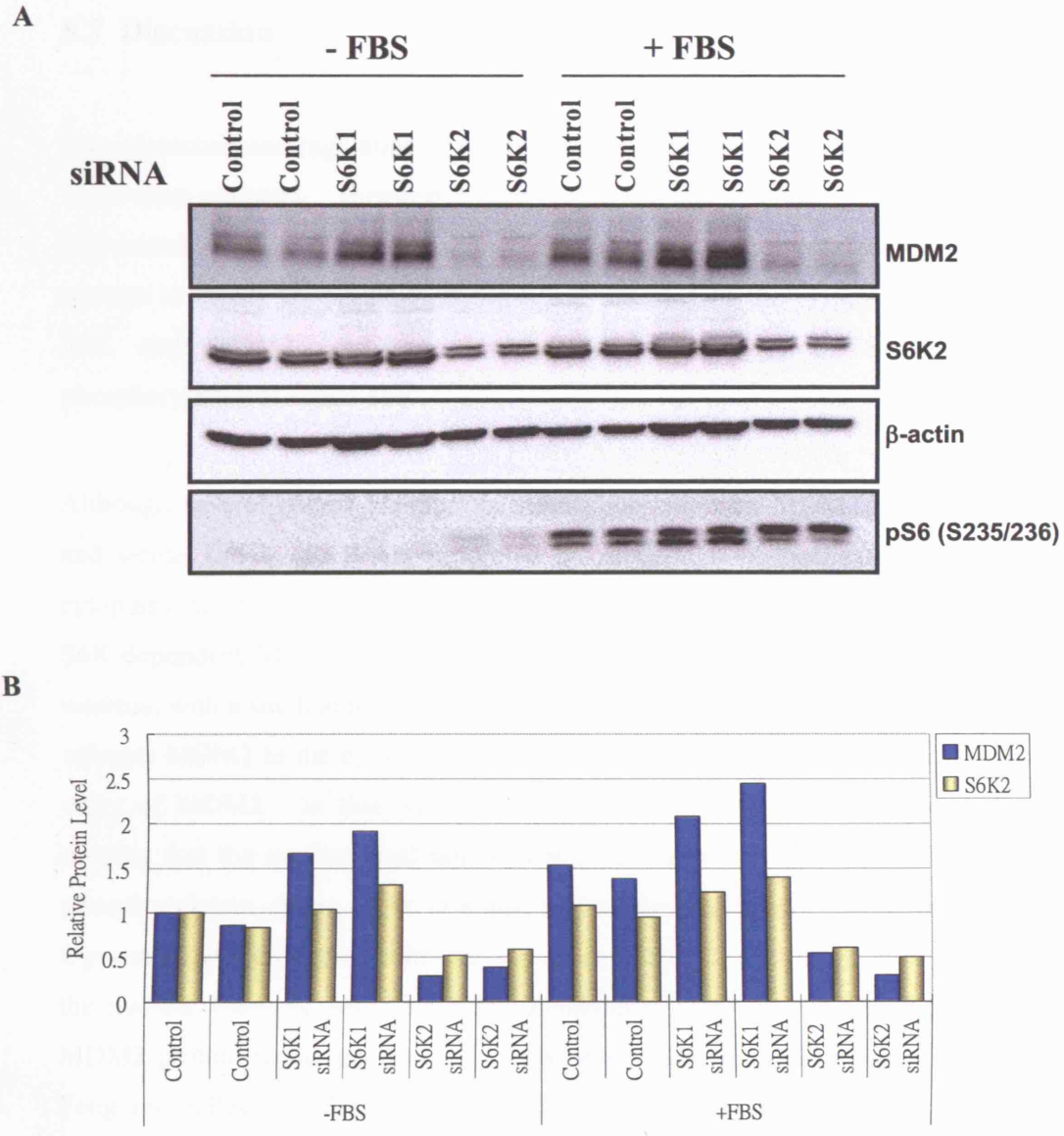

Fig. 5.15 The siRNA for S6K2 reduces MDM2 protein level.

A. HEK293 cells were seeded in 60 mm culture plates with the density of 25×10^4 cells per dish. Cells were transfected with 400 pM of either S6K1, S6K2, or non-specific control siRNA as indicated in the figure. Following 3 days of incubation in DMEM culture medium, cells were starved in serum-free DMEM medium for another 24 hours. After one-hour incubation with or without 10% FBS, the total cellular proteins were extracted with EB lysis buffer. Fifty microliter of each lysate was separated on SDS-PAGE and subjected to Western blotting using specific antibodies. **B.** The protein levels of MDM2 and S6K2 were quantified by densitometry and standardized with β-actin. Data was presented as a relative value to the control siRNA-transfected cells in non-stimulated panel. The same result was observed in duplicates in three independent experiments.

5.3 Discussion

The interaction and regulation of MDM2 through S6K activity has been described in previous chapters. However, the physiological significance of this interaction still remains to be fully understood. In this chapter, I presented my data in attempt to assess the possible physiological meaning of the association between S6K and MDM2, and the downstream consequences of S6K-mediated phosphorylation at serine 166.

Although several papers claimed that phosphorylation on MDM2 at serine 166 and serine 186 by PKB/Akt promoted its subcellular re-localization from the cytoplasm to the nucleus, my data showed that this was not true in the case of S6K-dependent MDM2 phosphorylation. MDM2 was mainly localized in the nucleus, with a small amount present in the cytoplasm. Serum starvation did not relocate MDM2 to the cytoplasm; neither did serum stimulation promote nucleus entry of MDM2. In line with my finding, several groups have reported data arguing that the nuclear localization of MDM2 might be independent of MDM2 phosphorylation status. For example, subcellular fractionation experiments by Ogawara and colleagues, indicated that more than 90% of MDM2 was found in the nuclear fraction; and LY294002 treatment did not change the amounts of MDM2 protein in the nuclear and cytoplasmic fractions (Ogawara *et al.*, 2002). Feng and colleagues also stated that the changes in MDM2 localization between the presence and absence of PKB/Akt was not observed in their experiments (Feng *et al.*, 2004). This disagreement indicates the complexity of MDM2 distribution regulation in cells. I do not exclude the regulation of MDM2 subcellular localization by PKB/Akt. In fact, it is conceivable that PKB/Akt may be different from S6K in regulating MDM2, since PKB/Akt mediates serine 186 phosphorylation while S6K phosphorylates MDM2 at serine 166. I suggest that S6K and PKB/Akt have divergent mechanisms by which they regulate MDM2 due to having two different phosphorylation sites on MDM2.

Unlike the controversial reports on subcellular localization, data on MDM2 stability seems to be more in agreement. The MDM2 protein level was clearly reduced by rapamycin, as well as LY294002, in my experiments. The reduction of MDM2 by both inhibitors was time- and dose-dependent, whereas rapamycin seems to have a stronger effect over MDM2 stability than LY294002. On the other hand, in agreement with the paper published by Ashcroft and colleagues (Ashcroft *et al.*, 2002), endogenous MDM2 half-life was reduced by 50% upon rapamycin and LY294002 treatment in MCF-7 cells. Moreover, substitution of the serine 166 with alanine was found to reduce MDM2 half-life in two different cell lines. These findings provide evidence that both S6K activity and phosphorylation of MDM2 at serine 166 are essential to maintain a stable cellular level of MDM2. Though my results seem to mirror that of previous publications, claiming that an active PI3K pathway, and upstream signalling of S6K, stabilized MDM2 by reducing its proteasomal degradation (Ashcroft *et al.*, 2002;Feng *et al.*, 2004), one aspect remains to be discussed. The observation that rapamycin is sufficient to reduce MDM2 protein level, indicates that the regulation of MDM2 stability may be mediated more directly through the mTOR/S6K pathway rather than PI3K pathway in some circumstances. This idea seems reasonable, taking into consideration my previous data which showed PKB/Akt as being insufficient to phosphorylate MDM2 at serine 166, an important feature of stabilizing the protein. Taken together, I suggest that S6K-mediated serine 166 phosphorylation protects MDM2 from the protein degradation pathway, resulting in a prolonged half-life of MDM2.

The first physiological function of MDM2 to be identified was as a negative regulator of p53 tumor suppressor. Recent discoveries of MDM2 as a downstream target for the PI3K pathway have disclosed an underlying mechanism by which the PI3K pathway negatively regulates p53-dependent apoptosis (Mayo and Donner, 2001;Ogawara *et al.*, 2002). Separate from reports about PKB/Akt and MDM2 interactions, my data indicated that S6Ks had no effect on p53 activity, nor did the serine 166 phosphorylation. Mutations on serine 166, which mimics unphosphorylated MDM2, did not restore p53 transactivation function; neither did

the active forms of S6K inhibit p53 activity, indicating that S6K-MDM2 signalling might lead to p53-independent regulation in cells. These results are reasonable when I take the subcellular localization data into account. Maya and colleagues claimed that PKB/Akt induces translocation of MDM2 into the nucleus and suppresses p53 transactivation functions (Mayo and Donner, 2001). This suppression of p53 is caused by MDM2-mediated degradation which is believed to take place in the cytoplasm (Mayo and Donner, 2001;Ogawara *et al.*, 2002). Therefore, the cytoplasm-nucleus shuttling becomes important for MDM2 to repress p53 transactivity. In my case, however, S6K has no effect on either MDM2 subcellular localization or p53 transactivity. Although MDM2 is mainly localized in the nucleus, it may not be capable of mediating p53 degradation and, consequently, inihibiting p53 transactivational function due to unchanged subcellular localization after serine 166 phosphorylation, which is mediated by S6K. These results imply that the S6K-dependent phosphorylation of MDM2 has a distinct regulatory pathway from that mediated by PKB/Akt, and the downstream effects of the S6K-MDM2 regulation could be independent of p53.

The cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$ is one of the prime transcriptional targets of p53 and mediates p53-dependent cell growth arrest and senescence. However, increasing number of data have shown that regulation of $p21^{waf1/cip1}$ may be more complicated than previously thought. Its transcription, as well as its cellular responses to stress signals or DNA damage, can be regulated through p53-dependent and –independent pathways (el Deiry *et al.*, 1994;Gartel and Tyner, 1999;Sato *et al.*, 2002;Liu *et al.*, 2003). Recently, MDM2 has been reported as a negative regulator of $p21^{waf1/cip1}$ independent of p53 (Sheaff *et al.*, 2000;Jin *et al.*, 2003;Zhang *et al.*, 2004), making $p21^{waf1/cip1}$ a suspected target for the S6K-MDM2 signalling pathway. My data showed that rapamycin increased $p21^{waf1/cip1}$ levels in the $p53^{-/-}$ PC-3 cells. In addition, cells presented higher levels of $p21^{waf1/cip1}$ when transfected with MDM2-166A. These results clearly indicate that the S6K-mediated MDM2 phosphorylation at serine 166 plays an important role in the regulation of $p21^{waf1/cip1}$ levels, independent of p53. This discovery is in line with previous reports, in which rapamycin has been shown to

increase $p21^{waf1/cip1}$ levels in human prostate cancer cell lines (Gao *et al.*, 2003). Gao and colleague suggested that mTOR and S6K are downstream of PI3K and PKB/Akt in regulating G1 cell cycle progression through mediating protein stability of $p21^{waf1/cip1}$ in the prostate cancer cells. In parallel with their findings, I have identified MDM2 as a downstream target of S6K that is involved in the regulation of $p21^{waf1/cip1}$ stability. Also, $p21^{waf1/cip1}$ appears to be degraded solely in the nucleus (Sheaff *et al.*, 2000), which implies that cytoplasm-nucleus shuttling may not be needed for degrading $p21^{waf1/cip1}$. Since S6K could not change MDM2 subcellular localization, this paper further strengthens my hypothesis. Furthermore, the finding of $p21^{waf1/cip1}$ downstream of the S6K-MDM2 signalling might reveal a possible explanation of how the mTOR-S6K signalling pathway regulates the cell cycle, and how rapamycin inhibits or delays cell cycle progression at G1/S transition.

Since the cloning of S6K2 (Gout *et al.*, 1998), publications have hinted at a link in the similarity between S6K1 and S6K2, rather than on their divergences. The knock out mice for either kinase was viable, and proposed overlapping functions of the two isoforms. The scarcity of publications on the difference between them is partially due to the absence of a practical experimental tool which is able to distinguish them. Rapamycin has long been the most commonly used inhibitor in mTOR/S6Ks signalling research. However, it is not capable of specifically inhibiting either form of S6Ks. Recently, reports on the divergent functions of S6K1 and 2 have begun to accumulate in the literature. Pardo and colleagues have presented an anti-apoptotic property of S6K2 (Pardo *et al.*, 2006), whereas Harrington and colleagues, by means of RNA interference, have demonstrated active S6K1 but not S6K2 ability of phosphorylating IRS-1, resulting in reduced signalling to the PI3K pathway (Harrington *et al.*, 2004). By using siRNAs against either S6K1 or S6K2, I have shown that the regulation of MDM2 is associated more with S6K2. Also, there seems to be a balancing regulation between the two isoforms. Severe reduction in S6K1 could cause an elevation in S6K2 expression. This regulation is unidirectional because repressed S6K2 have no effect on S6K1 expression.

CHAPTER SIX:

GENERAL DISCUSSION

CHAPTER SIX

GENERAL DISCUSSION

Cell cycle regulation is one of the known but poorly defined functions of the mTOR/S6K pathway. The slower proliferative rate of the embryonic stem cells lacking S6K1 (Kawasome *et al.*, 1998) and the rescue effect of rapamycin-resistance S6K1 on the rapamycin-induced delay in G1/S transition (Fingar *et al.*, 2004), imply a role for S6K1 on cell proliferation in a rapamycin-dependent manner. Although there exists several lines of evidence, as described in chapter one, suggesting the involvement of S6Ks in the signalling which regulates cell proliferation, the exact mechanism by which S6Ks mediate this regulation still remain to be discovered. This vague understanding of the function of S6Ks may be largely due to lack of a direct substrate linking it to cell cycle control.

The ribosomal S6 protein (rpS6) was the first, and for many years the only, substrate of S6Ks shown to undergo inducible phosphorylation, however, others have recently begun to emerge (Figure 1.8). At present, nine such substrates have been described, but for most there is limited or no information available regarding the effect of phosphorylation by S6K or other biological activity. In this thesis, I have demonstrated the discovery of a novel S6K substrate and attempted to address its physiological importance in normal and cancer cells.

The first impression of my research was derived from the reports on PKB/Akt-mediated phosphorylation on MDM2, a transcriptional target of p53 that negatively regulates p53. MDM2 was originally identified as an oncoprotein which binds to p53 and inhibits p53-mediated transactivation (Figure 1.10) (Momand *et al.*, 2000). The *mdm2* gene was found to be upregulated in human tumors and tumor cell lines by gene amplification, increased transcript levels, and

enhanced translation. The overall frequency of MDM2 amplification in human tumor tissue samples is approximately 7%, with the highest frequency observed in soft-tissue sarcomas (20 - 30%), osteosarcomas (16%), and oesophageal carcinomas (13%). However, simultaneous mutation of p53 and amplification of MDM2 does not generally occur within the same tumor (Momand *et al.*, 1998). The discovery of the regulation between MDM2 and PKB/Akt might explain the anti-apoptotic function of PKB/Akt and its role in transformation (Zhou *et al.*, 2001;Mayo and Donner, 2001;Ashcroft *et al.*, 2002;Ogawara *et al.*, 2002;Gottlieb *et al.*, 2002).

The fact that PKB/Akt and S6Ks share the same substrate recognition motif inspired us to investigate whether S6K is involved in MDM2 regulation. I began by examining protein-protein interactions. In actual fact, several proteins have been identified in various systems as binding partners of MDM2. These binding partners include ARF, L11, p300/CBP, PKB/Akt, ATM, CK2, HIF-1α, and many other upstream regulators that control MDM2 functions (Zhang *et al.*, 1998;Kamijo *et al.*, 1998;Chin *et al.*, 1998;Grossman *et al.*, 1998;Mayo and Donner, 2001;Kawai *et al.*, 2001;Ashcroft *et al.*, 2002;Lohrum *et al.*, 2003;Chen *et al.*, 2003). By using recombinant GST-fusion proteins, I have highlighted a direct interaction between MDM2 and S6K *in vitro* (Figure 3.3). This association is also present in three different cell lines, revealing S6K as a novel binding partner for MDM2 (Figure 3.4). Although the binding motif on MDM2 has not been identified, I suggest that the interaction could be more favorable with the carboxyl half of MDM2. Besides, the S6K-MDM2 interaction is severely reduced by rapamycin treatment indicating that the association is a rapamycin-sensitive event (Figure 3.6). Moreover, not only the S6K protein but also the activity of the protein, was found to be co-immunoprecipitated with MDM2 (Figure 3.7), suggesting S6K activity may be important for the association, or that MDM2 could be a substrate for S6K phosphorylation activity. In fact, I observed a preferred association of MDM2 with active S6K in HEK293 cells, but this phenomenon was not obvious in MCF-7 and U2OS cells (Figure 3.4). I reasoned that this inconsistency may have been caused by the varying responses

of different cell types to extracellular stimuli. Similar to my observations, conflicting results have been reported regarding the interaction between MDM2 and PKB/Akt.

Almost 20% of the amino acids in the MDM2 protein are consisted of either serine or threonine residues, and the MDM2 protein is phosphorylated at multiple sites *in vivo* (Hay and Meek, 2000). Phosphorylation sites on MDM2 have been mapped and reported during the last decade. Soon after the discovery of PKB/Akt and MDM2 interaction, two sites on MDM2 were identified as targets for PKB/Akt-mediated phosphorylation. Several reports claimed that Akt/PKB phosphorylates MDM2 at serine 166 and/or serine 186 and in turn, enhances its ability to promote p53 degradation (Zhou *et al.*, 2001;Mayo and Donner, 2001;Ashcroft *et al.*, 2002;Ogawara *et al.*, 2002;Gottlieb *et al.*, 2002;Feng *et al.*, 2004). As a downstream effector of PKB/Akt, I have shown that S6K mediates MDM2 phosphorylation *in vitro* (Figure 4.2), and the phosphorylation site on MDM2 has been mapped at serine 166, one of the PKB/Akt-mediated sites (Figure 4.3). The S6K-induced serine 166 phosphorylation was later confirmed in an S6 kinase assay using a MDM2 mutant where serine 166 was substituted into alanine (Figure 4.4). By employing a phospho-specific antibody against phosphorylated serine 166 on MDM2, I was able to show the S6K-mediated MDM2 phosphorylation *in vivo*.

Although the role of PKB/Akt on MDM2 phosphorylation has been reported in the literature (Zhou *et al.*, 2001;Mayo and Donner, 2001;Ashcroft *et al.*, 2002), my results indicate that S6K directly phosphorylates MDM2 at serine 166 rather than PKB/Akt on four counts. First of all, stimulating HEK293 and MCF-7 cells with different extracellular stimuli showed that some of the stimuli were capable of inducing serine 166 phosphorylation without activating PKB/Akt (Figure 4.6). This indicated that PKB/Akt activity may not be necessary for phosphorylation at serine 166. Instead, phosphorylation at serine 166 occurred in close parallel with that of threonine 389, an experimental readout of S6K activity, suggesting a rather direct link between the two proteins. Secondly, rapamycin dephosphorylated

serine 166 in a dose-dependent manner (Figure 4.8). As PKB/Akt activity is not an inhibitory target of rapamycin, this result implies that PKB/Akt may not be sufficient to mediate MDM2 phosphorylation at this site, which is a rapamycin-sensitive event. Thirdly, a rapamycin-resistant mutant of S6K, in which S6K is constitutively active independent of mTOR and insensitive to rapamycin, rescued the inhibitory effect of rapamycin on phospho-serine 166, proposed that S6K activity is necessary for inducing the phosphorylation (Figure 4.10). Lastly, amino acid depletion resulted in a reduction in the level of serine 166 phosphorylation, which was successively increased by the re-addition of essential amino acids (Figure 4.9). Since amino acids are one of the essential signals leading to mTOR/S6K but not the PI3K pathway, the result strongly indicates that serine 166 phosphorylation relies on the signals coming via the mTOR/S6K pathway, and that activation of the mTOR/S6K pathway is sufficient to induce serine 166 phosphorylation. Taken together, these results have led me to propose that the PKB/Akt activity appears neither essential nor sufficient to regulate MDM2 phosphorylation at serine 166, at least in some cases. Instead, S6K may play a major role on phosphorylation at this site. The MDM2 phosphorylation at serine 166 seems to function more as a downstream consequence of S6K rather than PKB/Akt. Considering previous reports on PKB/Akt-mediated MDM2 phosphorylation, there are contradicting reports in the literature. Some groups identified two sites, mapped at serine 166 and serine 186, on MDM2 as PKB/Akt targets (Zhou *et al.*, 2001;Mayo and Donner, 2001), whereas Ogawara and colleagues reported serine 186 only as PKB/Akt-recognized site (Ogawara *et al.*, 2002). Due to a regulatory link between PKB/Akt and S6K, it is possible that the PKB/Akt-mediated MDM2 phosphorylation is through active S6K. However, I have still not ruled out the possibility that, in some circumstances, PKB/Akt may play a direct role on phosphorylation at serine 166.

The increasing data on cellular proteins that associate with or post-translationally modify MDM2 suggests multiple functions of MDM2 in regulating cellular events, which includes inhibition of p53 and hence contribution to survival. For

example, DNA-PK phosphorylates MDM2 at serine 17 thus disrupting MDM2/p53 complex (Mayo *et al.*, 1997), while PKB/Akt-mediated phosphorylation results in the stabilization of MDM2 and reduction of p53 cellular levels (Mayo and Donner, 2001;Ogawara *et al.*, 2002). Reports on the PKB/Akt-mediated MDM2 phosphorylation claimed that it promotes nuclear entry of MDM2, where it binds to p53 and results in a rapid degradation of both proteins via proteasome pathway (Zhou *et al.*, 2001;Mayo and Donner, 2001;Ogawara *et al.*, 2002). However, nuclear localization seems not to be the consequence of S6K-mediated MDM2 phosphorylation. According to my data, the MDM2 protein was mainly located in the nucleus. Depletion of serum did not export MDM2 to the cytoplasm, nor did serum stimulation attract MDM2 back to the nucleus (Figure 5.1). In fact, the discovery that PKB/Akt mediates MDM2 sub-cellular localization, though well established, has been challenged by some groups. Similar to my observation, Ogawara *et al.* showed that 90% of MDM2 was in the nuclear fraction; serum starvation or LY294002 treatment did not alter it (Ogawara *et al.*, 2002). Similar results were also reported by Feng and colleagues, in which MDM2 is localized within the nucleus irrespective of the presence or absence of PKB/Akt-dependent phosphorylation. This disagreement indicates the complexity with which MDM2 distribution in cells is regulated.

In addition, the phosphorylation status at serine 166, based on my results, may not be important for p53 transcriptional activity. In my reporter systems, an MDM2 mutant containing an alanine substitution for serine 166, did not impede its inhibitory effect towards p53 (Figure 5.9); neither did active forms of S6Ks act to enhance it (Figure 5.10). This data indicates that neither S6K activity nor serine 166 phosphorylation has a detectable effect on p53 transcriptional activity. It is conceivable that S6K-mediated MDM2 phosphorylation had little effect on p53 activity since MDM2 subcellular localization, which is important for p53 degradation, was not changed by either active S6Ks or phosphorylated serine 166.

The stability of proteins in cells is an obvious factor in regulating the strength of their functions. As an inhibitor of an apoptosis pathway, MDM2 must be tightly

regulated, enabling cells to react to cellular stress and DNA damage fast enough to resume p53 function and turn on apoptosis signalling. It is well established that MDM2 functions are largely regulated by its stability. Ashcroft and colleague have reported a positive correlation between MDM2 stability and activation of the PI3K pathway (Ashcroft *et al.*, 2002). In agreement with their findings, MDM2 stability was sensitive to the PI3K inhibitor, LY294002, in my system. Interestingly, my results demonstrated clearly that S6K has a positive effect on MDM2 stability. First of all, a serum-induced elevation of cellular MDM2 level was observed under confocal microscope, though the subcellular localization of MDM2 was not changed, suggesting a serum-responding pathway is in charge of regulating MDM2 stability (Figure 5.1). Secondly, cellular MDM2 protein level was negatively regulated by rapamycin, as well as LY294002, in a dose- and time-dependent manner, indicating that MDM2 stability is managed in a rapamycin-sensitive pathway, such as mTOR/S6K (Figure 5.4-5.6). Thirdly, 50 nM of rapamycin efficiently reduced MDM2 half-life by 50% in the presence of CHX, providing evidence that the mechanism by which a rapamycin-sensitive pathway regulates MDM2 stability is probably through mediating protein turn-over rather than translation (Figure 5.7). Finally, the fact that the MDM2 mutant containing a substitution at serine 166 with alanine had a shorter half-life than wild-type MDM2, suggests that S6K exerts its positive effect on MDM2 stability through phosphorylation at serine 166 (Figure 5.8). These data taken together shed some light on the physiological importance of the S6K-mediated MDM2 phosphorylation process. I therefore concluded that S6K may upregulate MDM2 by reducing its proteosomal degradation which involved its phosphorylation at serine 166.

MDM2 has been reported to have p53-independent functions, in cell cycle control, differentiation, cell fate determination, DNA repair, basal transcription, and other processes. As described earlier in this chapter, S6K-MDM2 signalling may have no effect on p53-dependent regulatory pathway, which is the reason I have concentrated my research on the search for a downstream effector of MDM2, with which the signalling from S6K could be transducted into a physiological

regulation, independently of cellular p53 status. The first attempt at searching for a downstream target of MDM2, focussed on the nuclear phosphoprotein, Rb, since I observed a correlation between the decrease of MDM2 protein level and the decrease of hyperphosphorylated Rb under the influence of rapamycin. In line with my findings, studies carried out by Gao *et al.,* also implied a role for the rapamycin-sensitive pathway on the regulation of Rb phosphorylation (Gao *et al.*, 2003;Gao *et al.*, 2004). However, further investigations seemed to separate Rb from S6K-MDM2 signalling. Both overexpressed S6K1 and MDM2-166A mutants had little effect on Rb function, using an Rb-reporter assay (data not shown). The introduction of excess S6K1 in cells did not reduce Rb activity, nor did MDM2-166A mutants impair its inhibitory effect on Rb. It is more likely that other rapamycin-sensitive signaling(s), instead of the S6K-MDM2 pathway, take part in Rb regulation.

One of the potential candidates for a MDM2 downstream effector might be the cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$. Although it is primarily considered as a transcriptional target of p53, $p21^{waf1/cip1}$ transcription can be regulated via either p53-dependent (el Deiry *et al.*, 1994) or –independent (Gartel and Tyner, 1999;Sato *et al.*, 2002) pathways. Albeit that $p21^{waf1/cip1}$ activation is important in mediating p53-dependent cell growth arrest, it is not essential for p53-mediated apoptosis (Deng *et al.*, 1995). MDM2 has been shown to mediate proteasomal degradation of $p21^{waf1/cip1}$ independent of p53 and ubiquitination, and thus negatively affected $p21^{waf1/cip1}$–mediated cell growth arrest (Jin *et al.*, 2003;Zhang *et al.*, 2004). In line with previous publications (Gao *et al.*, 2003), I have shown in the present study that rapamycin increased the cellular level of $p21^{waf1/cip1}$ in prostate cancer cells (Figure 5.11). Moreover, the MDM2-166A but not MDM2-186A mutant lost its inhibitory effect on $p21^{waf1/cip1}$ protein level, suggesting a key role for site-specific phosphorylation at serine 166 (Figure 5.12). These data implied that a rapamycin-sensitive pathway is involved in $p21^{waf1/cip1}$ regulation, and the regulation is in a p53-independent fashion because the p53-null PC-3 cell line was used in these experiments.

Though further investigations may be needed to clarify the role of $p21^{waf1/cip1}$ in S6K-MDM2 signalling, some interesting evidence exists in support of this observation. First of all, rapamycin has been shown to have a positive effect on cellular $p21^{waf1/cip1}$ level in variant prostate cancer cell lines whatever the status of cellular p53 is (Gao *et al.*, 2003). Secondly, the inhibition of MDM2 by antisense oligonucleotides, elevated $p21^{waf1/cip1}$ levels in breast and prostate cancer cell lines, regardless of p53 (Wang *et al.*, 2001a;Zhang *et al.*, 2003). Finally, the discovery by Jin *et al.* that the amino acid 151-229 of MDM2 is essential for the association between MDM2 and $p21^{waf1/cip1}$, which is one of the pre-requisites of $p21^{waf1/cip1}$ degradation (Jin *et al.*, 2003), further supporting a role of serine 166 phosphorylation in MDM2-mediated $p21^{waf1/cip1}$ regulation. Taking these results together, I suggested that $p21^{waf1/cip1}$ may play a role downstream of the S6K-MDM2 signalling pathway, a signal from which inhibits $p21^{waf1/cip1}$ and enhances cell survival independent of p53. The exact mechanism by which MDM2, especially serine 166-phosphorylated MDM2, regulates $p21^{waf1/cip1}$ still remains elusive; one possibility is that phosphorylation at serine 166 might creates a binding-friendly form of MDM2 for $p21^{waf1/cip1}$, which is in turn directed to proteasomal degradation. Nevertheless, the increased $p21^{waf1/cip1}$ might explain, at least in part, the impaired development and survival of S6K-defecient *Drosophila* and mice (Montagne *et al.*, 1999;Pende *et al.*, 2004).

There are still potential substrates for S6K-MDM2 signalling which remain to be investigated, including hypoxia-inducible factor 1 (HIF-1). HIF-1 is a heterodimeric transcription factor composed of HIF-1α and HIF-1β subunits. HIF-1β is constitutively expressed in cells, whereas HIF-1α expression is up-regulated by hypoxia, as well as by a variety of growth factors and oncogenes. HIF-1 activity is believed to be regulated primarily by the levels of HIF-1α in cells. HIF-1α has previously been shown to play a crucial role in both angiogenesis and tumor growth (Maxwell *et al.*, 1997a;Ryan *et al.*, 1998;Kung *et al.*, 2000b;Ryan *et al.*, 2000b). Inhibition of HIF-1α expression leads to decreased tumor size *in vivo*, whereas increased HIF-1α expression has the reverse effect (Maxwell *et al.*, 1997b;Kung *et al.*, 2000a;Ryan *et al.*, 2000a). In

some cancers, HIF-1α expression is associated with tumor aggressiveness and patient mortality (Birner *et al.*, 2000;Zagzag *et al.*, 2000;Birner *et al.*, 2001;Bos *et al.*, 2001).

Both mTOR/S6K and MDM2/p53 signalling pathways have been individually shown to regulate HIF-1α expression in some cell systems in response to growth factors and hypoxia. Rapamycin negatively regulated HIF-1α protein expression (Skinner *et al.*, 2004), whereas overexpressed S6K1 increased the protein level of HIF-1α and its primary transcriptional target, VEGF (Skinner *et al.*, 2004;Fang *et al.*, 2005a). It has been shown that forced expression of MDM2 was able to elevate HIF-1α protein level (Fang *et al.*, 2005c), and restore LY294002-inhibited VEGF protein production (Skinner *et al.*, 2004). Fang *et al.* suggested that blockage of MDM2 phosphorylation, as well as destabilization of the protein, could be a possible mechanism through which apigenin, an antitumor reagent, reduces HIF-1α expression (Fang *et al.*, 2005b). Although these reports claimed that S6K and MDM2 are two parallel pathways which mediate growth factor-induced HIF-1α expression, the direct link between S6K and MDM2 on regulating HIF-1α has not yet been explored. Considering my discoveries on S6K-MDM2 regulation, a linear model may still exist where HIF-1α is located downstream of MDM2 in response to S6K-mediated phosphorylation. It would be interesting to find out whether or not MDM2 is able to recover rapamycin-inhibited HIF-1α expression, as well as using short interfering RNA against MDM2 to determine the signalling network upstream of HIF-1α, especially after the discovery of direct interaction between MDM2 and HIF-1α (Chen *et al.*, 2003). Additional studies should elucidate the potential interaction between HIF-1α and the S6K-MDM2 signalling.

Specific inhibition of either isoform of S6K by employing RNA interfering techniques have recently revealed functional divergences between S6K1 and 2 (Harrington *et al.*, 2004;Pardo *et al.*, 2006). It seems that S6K1 is more closely related to the TSC-mediated inhibition of mTOR/S6K signalling, and the negative

effect on IRS-1 (Harrington *et al.*, 2004), while S6K2 has been reported to have an anti-apoptotic function (Pardo *et al.*, 2006). My experiments with small interfering RNA against either S6K isoform showed that S6K2 might be more prevalent in contributing to MDM2 regulation in some circumstances (Figure 5.14-5.15), although both kinases were capable of MDM2 phosphorylation. S6K2 complemented the cellular protein level of S6K1, as well as its function of phosphorylating and stabilizing MDM2, when the level of S6K1 was severely repressed by siRNA. However, this complementary effect did not apply to siRNA-induced reduction of S6K2, which could indicate diversity between regulating the two isoforms and the unique nature of some cellular functions which cannot be replaced by the other isoform.

Cancer cells often exhibit alterations in the signal transduction pathways leading to proliferation in response to external signals. The contribution of S6K on cell cycle progression and cell proliferation has been described in chapter one. Although rapamycin analogues have shown a promising anti-tumor effect in several types of refractory tumors and are seemingly well tolerated by patients in Phase I trials, only some patients appear to respond to this treatment. Mounting evidence has implied that rapamycin resistance may occur by multiple mechanisms. Decreased binding of rapamycin to the target mTOR may be the cause of resistance to rapamycin. Mutations or defects of downstream effector molecules of mTOR, such as S6K, PP2A-related phosphotase, or 4E-BP1, also protect cells from inhibition by rapamycin. My study in breast cancer cell lines may highlight the role of MDM2 in the mechanism of rapamycin resistance. During treatment with rapamycin, the serine 166 phosphorylation of MDM2 was inhibited in rapamycin-sensitive cells but retained in resistant cells, indicating the existence of a mechanism which maintained the phosphorylation of serine 166 in resistant cells, even under the effect of rapamycin. Interestingly, S6K activity toward ribosomal S6 protein was sensitive to rapamycin in both cell lines, indicating divergent regulations on MDM2 and ribosomal S6 protein in rapamycin-resistant cell lines. The exact mechanism by which phosphorylation of serine 166 resists the inhibitory effect of rapamycin is still elusive. One

possible explanation could be that cyclin G, is shown to stimulate the ability of PP2A to dephosphorylate MDM2 at serine 166 (Okamoto *et al.*, 2002). Further experiments are needed to explore the role of cyclin G in rapamycin-resistance mechanisms. Moreover, the failure of rapamycin to dephosphorylate the nucleus form of S6K1 in rapamycin-resistant cell lines has further complicated the mechanism of resistance to rapamycin.

The MDM2 protein is overexpressed in a significant number of human tumors underscoring its pivotal involvement in the development of human disease. My result has also shown a proliferative advantage of serine 166-phosphorylated MDM2 in stable cell lines. The anti-apoptotic property of MDM2 has made itself an interesting target for cancer therapy. Laboratory data with antisense anti-MDM2 oligonucleotide, has provided promising results on repressing tumor growth via inhibition of MDM2 (Wang *et al.*, 2003). Through combination with other chemotherapeutic agents, it increased therapeutic effectiveness and reduced tumor growth in mice. Despite its unsolved mechanism, the discovery of distinct responses of serine 166 phosphorylation to rapamycin between sensitive and resistant cell lines, have suggested that phosphorylation of MDM2 may be considered as a molecular marker to determine and prognose cellular response to rapamycin. It would be interesting to examine whether repressing serine 166 phosphorylation in resistant cell lines could restore its sensitivity to rapamycin on cell growth and proliferation.

In conclusion, in the present study I have identified a novel downstream target for S6K, the oncoprotein MDM2. MDM2 is directly associated with and phosphorylated by S6K *in vitro* and *in vivo*. The phosphorylation site for S6K has been identified as serine 166, which was previously reported as a PKB site, but proved in this study to be directly phosphorylated by S6K rather than PKB in some circumstances, if not all. Unlike PKB, the S6K-mediated phosphorylation at serine 166 has no effect on either its subcellular localization or p53 activity. Instead, serine 166 phosphorylation enhanced its protein stability and inhibited cellular $p21^{waf1/cip1}$ level regardless of p53. Moreover, MDM2 phosphorylation

at serine 166 seemed to be involved in cell proliferation and to play a role on the mechanism of resistance to rapamycin. The discovery of S6K-MDM2 regulation may highlight a route by which the mTOR/S6K pathway controls cell proliferation and cell cycle progression, and may also partially explain the contribution of unregulated S6K activity to tumorigenesis. A better understanding of the molecular mechanisms involved in tumor progression will allow for the development of specific therapeutic agents to treat tumors.

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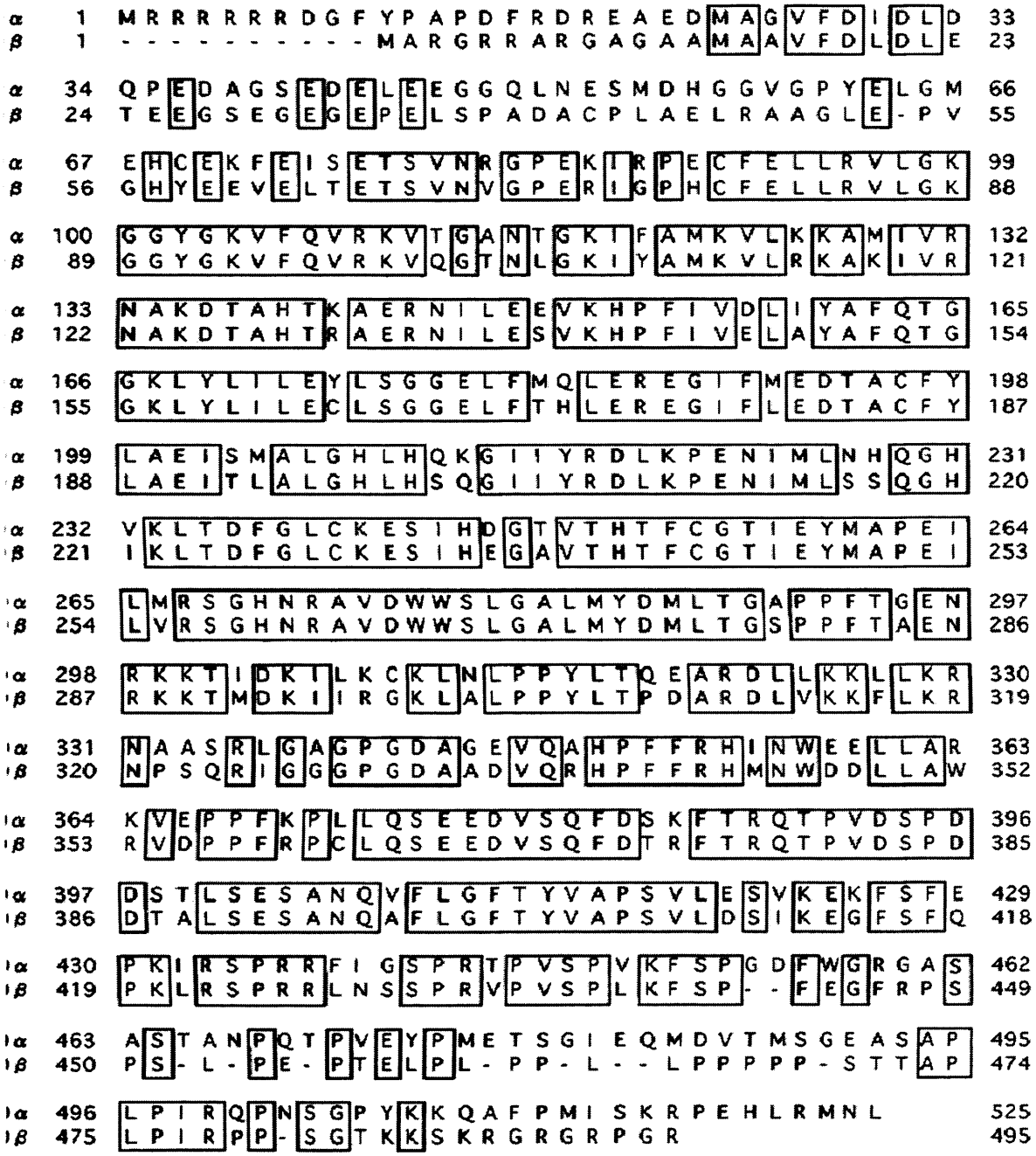
Appendix A



Comparison of S6K1 (α) with S6K2 (β).

Amino acid sequences of S6K1 and S6K2 are aligned. Conserved residues are boxed.

Appendix B

Mouse MDM2

MCNTNMSVST EGAASTSQIP ASEQETLVRP KPLLLKLLKS
VGAQNDTYTM KEIIFYIGQY IMTKRLYDEK QQHIVYCSND
LLGDVFGVPS FSVKEHRKIY AMIYRNLVAV SQQDSGTSLS
ESRRQPEGGS DLKDPLQAPP EEKPSSSDLI SRLSTSSRRR
SISETEENTD ELPGERHRKR RRSLSFDPSL GLCELREMCS
GGTSSSSSSS SESTETPSHQ DLDDGVSEHS GDCLDQDSVS
DQFSVEFEVE SLDSEDYSLS DEGHELSDED DEVYRVTVYQ
TGESDTDSFE GDPEISLADY WKCTSCNEMN PPLPSHCKRC
WTLRENWLPD DKGKDKVEIS EKAKLENSAQ AEEGLDVPDG
KKLTENDAKE PCAEEDSEEK AEQTPLSQES DDYSQPSTSS
SIVYSSQESV KELKEETQHK DESVESSFSL NAIEPCVICQ
GRPKNGCIVH GKTGHLMSCF TCAKKLKKRN KPCPVCRQPI
QMIVLSYFN

Human MDM2

MCNTNMSVPT DGAVTTSQIP ASEQETLVRP KPLLLKLLKS
VGAQKDTYTM KEVLFYLGQY IMTKRLYDEK QQHIVYCSND
LLGDLFGVPS FSVKEHRKIY TMIYRNLVVV NQQESSDSGT
SVSENRCHLE GGSDQKDLVQ ELQEEKPSSS HLVSRPSTSS
RRRAISETEE NSDELSGERQ RKRHKSDSIS LSFDESLALC
VIREICCERS SSSESTGTPS NPDLDAGVSE HSGDWLDQDS
VSDQFSVEFE VESLDSEDYS LSEEGQELSD EDDEVYQVTV
YQAGESDTDS FEEDPEISLA DYWKCTSCNE MNPPLPSHCN
RCWALRENWL PEDKGKDKGE ISEKAKLENS TQAEEGFDVP
DCKKTIVNDS RESCVEENDD KITQASQSQE SEDYSQPSTS
SSIIYSSQED VKEFEREETQ DKEESVESSL PLNAIEPCVI
CQGRPKNGCI VHGKTGHLMA CFTCAKKLKK RNKPCPVCRQ
PIQMIVLTYF P

Amino acid sequence of mouse and human MDM2.

The amino acid sequences of mouse and human MDM2 are listed above. The serine residue at amino acid 166 of human MDM2 is presented with shadow background.